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ANEMIA AND HIGH THERAPEUTIC DOSES OF RECOMBINANT HUMAN ERYTHROPOIETIN IN CHRONIC KIDNEY DISEASE – A LINKAGE OF RISK?

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Declara-se que fazem parte integrante desta tese os seguintes trabalhos já publicados ou em publicação:

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“Cada um cumpre o destino que lhe cumpre,
E deseja o destino que deseja;
Nem sempre cumpre o que deseja,
Nem deseja o que cumpre.”

Fernando Pessoa

Aos meus pais e ao meu irmão

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RESUMO

A anemia é uma complicação comum da doença renal crónica (DRC), cuja principal causa é a produção insuficiente de eritropoietina (EPO) pelos rins. A introdução dos agentes estimuladores da eritropoiese (AEE) revolucionou o tratamento da anemia da DRC; porém, alguns doentes não respondem adequadamente a esta terapia, necessitando de doses mais altas para atingir os níveis alvo de hemoglobina. Vários ensaios clínicos evidenciaram um aumento de eventos cardiovasculares e de mortalidade em doentes tratados com AEE, particularmente nos não respondedores, permanecendo por esclarecer as causas e os mecanismos subjacentes. Vários efeitos pleiotrópicos, para além da correção da anemia, têm sido atribuídos à EPO e aos AEE, tais como renoproteção e propriedades anti-inflamatórias; contudo, para obter estes efeitos são necessárias doses mais elevadas, cujos riscos-benefícios não foram ainda suficientemente estudados e definidos.

O nosso trabalho teve como objectivos i) estudar o desenvolvimento da anemia, a função e lesão renal, a pressão arterial, o metabolismo do ferro e a relação entre as diferentes alterações, usando como modelo animal de insuficiência renal crónica (IRC) o rato, submetido a nefrectomia cirúrgica de 5/6; ii) avaliar o impacto da terapêutica com eritropoietina humana recombinante (rHuEPO), tratando os animais com IRC, durante 3 semanas, com doses usadas para corrigir a anemia nos doentes com DRC (100 e 200 UI/peso (Kg)/semana), e com doses mais altas (400 e 600 UI/peso (Kg)/semana), usadas no tratamento de doentes não respondedores; iii) analisar os efeitos das mesmas doses de rHuEPO, *per se*, usando ratos saudáveis, sem IRC.

Os ratos com IRC não tratados desenvolveram anemia, hipertensão, inflamação (sistémica e renal) e lesões renais severas. O tratamento com rHuEPO nos animais com IRC corrigiu a anemia de forma dose-dependente. As doses mais baixas (grupos IRC100 e IRC200) melhoraram as lesões renais glomerulares e tubulointersticiais (apesar de ainda presentes), e reduziram os marcadores inflamatórios no tecido renal, mas sem impacto na inflamação sistémica (valores de PCR). As doses mais altas (IRC400 e IRC600) melhoraram substancialmente todas as lesões renais e a inflamação (renal e sistémica); todavia, a taxa de filtração glomerular manteve-se inalterada. Observou-se uma redução da pressão arterial em todos os animais tratados com rHuEPO, apesar de se ter verificado um aumento dose-dependente. Em ratos saudáveis, o tratamento com rHuEPO induziu um aumento dos eritrócitos, dose-dependente, aumentando a viscosidade sanguínea. O aumento da pressão arterial induzida pelo tratamento com rHuEPO pode resultar de uma ação direta desta nos vasos renais e/ou da hiperviscosidade sanguínea devido à eritrocitose, acompanhada por uma redução da atividade da enzima monóxido de azoto

sintetase endotelial. Nos grupos com pressão arterial mais elevada foram observadas lesões renais vasculares e tubulointersticiais.

Apesar do contexto inflamatório, não foram observadas alterações nos marcadores séricos do metabolismo do ferro. A hipóxia resultante da anemia no grupo não tratado e a eritropoiese estimulada com a dose mais baixa (IRC100) parecem determinantes na repressão da transcrição da hepcidina. As doses mais altas (IRC200 e IRC400) induziram um estímulo eritropoiético maior, aumentando a absorção de ferro de forma a disponibilizar ferro suficiente para a eritropoiese, determinando a inibição da síntese de hepcidina. A dose mais elevada (IRC600) promoveu um estímulo eritropoiético e uma absorção de ferro ainda superiores, induzindo acumulação de ferro hepático capaz de ativar a síntese de hepcidina através da via BMP6/SMAD. Nos ratos saudáveis, contrastando com os resultados obtidos nos animais com IRC, as doses mais altas não estimularam a produção de hepcidina; em vez disso, as doses mais altas reprimiram a síntese de hepcidina, apesar do aumento da absorção de ferro, sugerindo uma supremacia da eritropoiese sobre o ferro na regulação da hepcidina.

Finalmente, após uma correção inicial da anemia, um grupo de ratos tratados com rHuEPO (200 UI/peso (Kg)/semana) deixou subitamente de responder ao tratamento, desenvolvendo anemia. Nestes animais, observou-se um aumento dos níveis séricos de PCR e dos marcadores renais de inflamação (NF-κB, IL-1β and IL-6), contribuindo para a inibição da eritropoiese e da síntese renal de EPO. Além disso, observou-se um aumento dos marcadores de fibrose (CTGF e TGF-β1) no rim, contribuindo para fibrose renal.

Em conclusão, os resultados sugerem que um tratamento de curta duração com doses altas, para ultrapassar um episódio de resistência à terapia com rHuEPO, pode apresentar benefícios, por reduzir a inflamação; contudo, o efeito pro-hipertensor durante um tratamento de longa duração, particularmente com doses altas, deve ser devidamente monitorizado, para evitar um impacto cardiorrenal deletério. A correção da anemia com doses de rHuEPO mais elevadas, ao estimular a eritropoiese e a absorção de ferro, pode aumentar os níveis de ferro armazenados, induzindo a síntese de hepcidina, contribuindo assim para o desenvolvimento de resistência à terapia. A resistência à rHuEPO associada à inflamação agrava a anemia, favorecendo a hipóxia e a fibrose renal, que exacerbam ainda mais a resposta inflamatória, num ciclo vicioso que conduz a progressão da doença renal.

Palavras-chave: anemia; doença renal crónica; eritropoietina; hepcidina; hipertensão

ABSTRACT

Anemia is a common complication in chronic kidney disease (CKD) patients and its main cause is the insufficient production of erythropoietin (EPO) by the failing kidneys. The introduction of erythropoiesis-stimulating agents (ESA) revolutionized the treatment of anemia in CKD; however, some patients do not respond properly to ESA therapy requiring higher doses to achieve target hemoglobin (Hb) levels. Several clinical studies reported an increase in cardiovascular events and mortality in patients treated with ESA, especially in hyporesponsive patients, whose specific causes and mechanisms remain to be elucidated. Several pleiotropic effects, beyond anemia correction, have been attributed to EPO and ESA, such as renoprotection and anti-inflammatory properties, but to attain those effects higher ESA doses are needed, whose risk-benefits are still undisclosed, requiring further clarification.

Our work aimed i) to characterize anemia development, renal function and damage, blood pressure, iron metabolism and the crosstalk between the induced disturbances, using the remnant kidney rat model of chronic renal failure (CRF) induced by 5/6 surgical nephrectomy; ii) to evaluate the impact of different recombinant human erythropoietin (rHuEPO) therapy, by treating the CRF animals, during 3 weeks, with standard rHuEPO doses, usually used to correct anemia in CKD patients (100 and 200 IU/Kg body weigh [BW]/week), and with higher doses (400 and 600 IU/Kg BW/week), used in the treatment of hyporesponsive patients; iii) to dissect the effects of these rHuEPO doses, per se, using normal rats, without CRF.

We found that the untreated CRF group developed anemia, hypertension, inflammation (systemic and renal) and severe renal lesions. The use of rHuEPO in the CRF rats was able to correct the anemia in a dose-dependent manner. The lower doses tested (CRF100 and CRF200 groups) were able to ameliorate (but still present) glomerular and tubulointerstitial renal lesions, to reduce renal tissue inflammatory markers, without impact on systemic inflammation (CRP levels). The higher rHuEPO doses (CRF400 and CRF600 groups) markedly improved all renal lesions and inflammation (renal and systemic); however, glomerular filtration rate did not change. Blood pressure was reduced in all rHuEPO treated groups, even though a dose-dependent raise was found. In normal rats, rHuEPO treatment was able to induce a dose-dependent increment in erythrocytes, leading to increased blood viscosity. Thus, the rHuEPO-induced blood pressure raise might result from a direct effect on the renal vessels and/or from blood hyperviscosity associated with erythrocytosis, accompanied by a reduced activity of endothelial nitric oxide synthase. Vascular and tubulointerstitial renal lesions were presented by the groups with the highest blood pressure.

Despite the inflammatory state, no significant changes in serum iron markers were observed in untreated CRF rats. The hypoxia associated to anemia in the CRF group and the stimulated erythropoiesis with the lowest rHuEPO dose (CRF100) seems to prevail in the down-regulation of hepcidin mRNA levels. Higher rHuEPO doses (CRF200 and CRF400) induced a higher erythropoietic stimulus, increasing iron absorption to maintain iron availability for erythropoiesis, thus causing hepcidin synthesis repression. The highest rHuEPO dose (CRF600) endorsed an even higher erythropoietic stimulus and iron absorption, leading to liver iron accumulation, which is able to trigger hepcidin transcription through the BMP6/SMAD pathway. In healthy rats, contrasting with the results obtained in the CRF animals, higher rHuEPO doses were unable to induce an increase in hepcidin synthesis; instead, we found a down-regulation of hepcidin transcription with the highest doses, despite the increased iron absorption, suggesting the supremacy of erythropoiesis over iron in the regulation of hepcidin.

Finally, after an initial correction of anemia, a group of rats receiving rHuEPO therapy (200IU/Kg BW/week), presented a sudden hyporesponse to treatment, developing anemia. In these animals, serum CRP levels and renal inflammatory markers (NF-κB, IL-1β and IL-6) increased, contributing to suppress erythropoiesis and renal EPO synthesis. Furthermore, kidney fibrosis markers (CTGF and TGF-β1) were overexpressed, contributing to renal fibrosis.

In conclusion, our data suggest that a short-term treatment with high doses, used to overcome an episode of hyporesponse to rHuEPO, can present benefits by reducing inflammation, without worsening of renal lesions; however, the pro-hypertensive effect during a long-term treatment, particularly with higher rHuEPO doses, should be carefully managed in order to avoid a negative cardiorenal impact. Anemia correction with high rHuEPO doses, by favoring erythropoiesis and iron absorption, can increase iron stores, inducing hepcidin synthesis, which might contribute to hyporesponsiveness to rHuEPO therapy. This condition is associated with inflammation, aggravating anemia and favoring hypoxia and renal fibrosis, which further enhance the inflammatory response in a vicious cycle that promotes renal disease progression.

Keywords: anemia, chronic kidney disease, erythropoietin, hepcidin, hypertension

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LIST OF ABREVIATIONS

βCR	Common beta receptor
ACE	Angiotensin-converting-enzyme
ACR	Albumin-to-creatinine ratio
AER	Albumin excretion rate
Apo (a)	Apolipoprotein (a)
BFU-E	Burst-forming unit-erythroid
BMP6	Bone morphogenetic protein 6
BP	Blood pressure
BUN	Blood urea nitrogen
BW	Body weight
CFU-E	Colony-forming unit-erythroid
CKD	Chronic kidney disease
CHOIR	Correction of Hemoglobin and Outcomes in Renal Insufficiency
CREATE	Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta
CRF	Chronic renal failure
CRP	C-reactive protein
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DcytB	Duodenal cytochrome B
DMT1	Divalent metal iron transporter 1
EBPG	European Best Practice Guidelines
ECM	Extracellular matrix
EMA	European Medicines Agency
EMT	Epithelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ERK	Extracellular signal-Regulated Kinase
ESA	Erythropoiesis-stimulating agents
ESRD	End-stage renal disease
FDA	Food and Drug Administration
Fe	Iron
FIH	Factor-inhibiting-HIF

FPN	Ferroportin
GFR	Glomerular filtration rate
Hb	Hemoglobin
HCP-1	Heme carrier protein 1
HD	Hemodialysis
HDLc	High density lipoprotein cholesterol
HFE	Hemochromatosis
HIF	Hypoxia-inducible factor
HJV	Hemojuvelin
HO-1	Heme-oxygenase-1
HR	Heart rate
Ht	Hematocrit
IFN-γ	Interferon gamma
IFTA	Interstitial fibrosis and tubular atrophy
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IV	Intravenous
JAK	Janus Kinase
KDIGO	Kidney Disease Improving Global Outcomes
KW	Kidney weight
LDLc	Low density lipoprotein cholesterol
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
MBP	Mean blood pressure
MCV	Mean cell volume
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MW	Molecular weight
ND	Non-dialysis
NF-κB	Nuclear factor kappa B
NKF KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
NO	Nitric oxide
NTBI	Non-transferrin bound iron
PHD	Prolyl-4- hydroxylase domain
PTH	Parathyroid hormone
RBC	Red blood cells

REPC	Renal erythropoietin-producing cells
rHuEPO	Recombinant human erythropoietin
RAAS	Renin-angiotensin-aldosterone system
RPI	Reticulocyte production index
SBP	Systolic blood pressure
SMAD	Son of mothers against decapentaplegic
STAT	Signal transducer and activator of transcription
sTfR	Soluble transferrin receptors
Tf	Transferrin
Tf-Fe	Transferrin-bound iron
TfR	Transferrin receptor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TREAT	Trial to Reduce Cardiovascular Events with Aranesp Therapy
TSAT	Transferrin saturation
VEGF	Vascular endothelial growth factor
VHL	von Hippel–Lindau tumor suppressor protein
VSMC	Vascular smooth muscular cells
WBC	White blood cells

INTRODUCTION

1. CHRONIC KIDNEY DISEASE

1.1. Definition and classification

Chronic kidney disease (CKD) is a pathological condition that results from a gradual, permanent loss of kidney function over time, usually, months to years. According to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) guidelines, the diagnosis of CKD should be based on the presence of kidney damage or reduction of kidney function (evaluated by glomerular filtration rate – GFR), allowing to classify the disease in 5 stages (Table 1), with increasing severity [1].

Table 1 – Stages of chronic renal disease severity

Stage	GFR	Description
1	≥90	Kidney damage (protein in the urine) and normal GFR
2	60 - 89	Kidney damage and mild decrease in GFR
3	30 - 59	Moderate decrease in GFR
4	15 - 29	Severe decrease in GFR
5	<15	Kidney failure (dialysis or kidney transplant needed)

Abbreviations: GFR – Glomerular Filtration Rate (mL/min/1.73m²). Stage 3 can be subdivided in 3a (GRF 45–59, mildly to moderately decreased) and 3b (GFR 30–44, moderately to severely decreased). Adapted from the National Kidney Foundation (2002) [1].

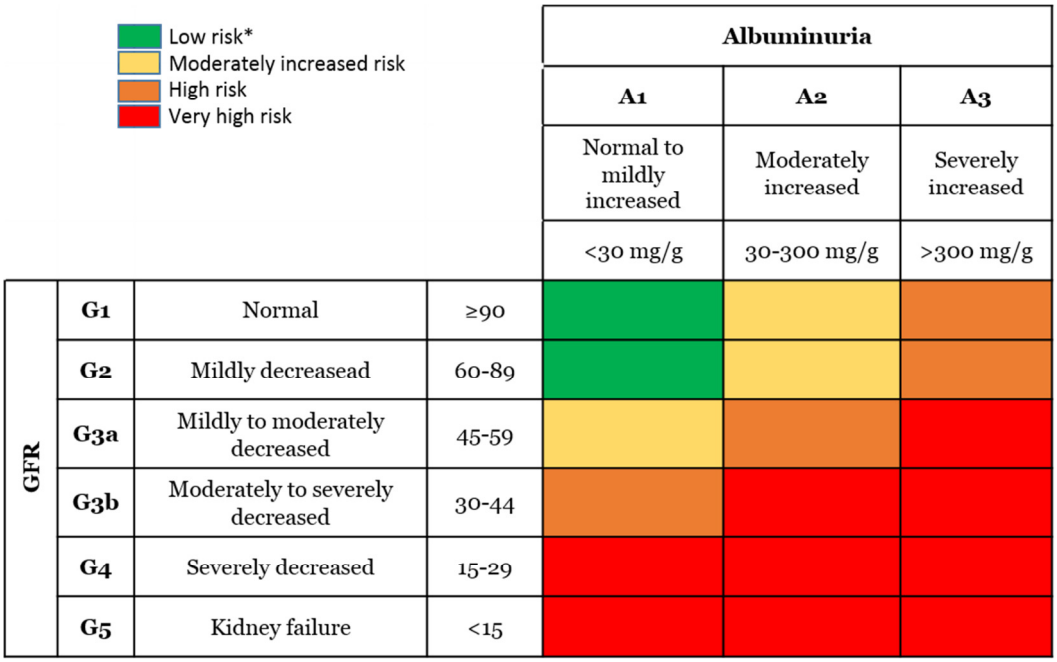
CKD is a complex pathology and, in the recent years, some updates to better classify CKD have been made. The recent Kidney Disease Improving Global Outcomes (KDIGO) guidelines for "Evaluation and Management of Chronic Kidney Disease" introduced albuminuria categories as a further criteria to CKD staging (Table 2) and risk prediction (Figure 1) [2].

Table 2 – Albuminuria categories in chronic kidney disease

Category	AER	ACR		Description
	(mg/24h)	(mg/mmol)	(mg/g)	
A1	< 30	< 3	< 30	Normal to mildly increased
A2	30 – 300	3 – 30	30 - 300	Moderately increased
A3	> 300	> 30	> 300	Severely increased

Abbreviations: ACR - albumin-to-creatinine ratio; AER - albumin excretion rate. Adapted from KDIGO Group (2012) [2].

CKD is confirmed if in a period superior to 3 months there is a decrease in kidney function (GFR < 60 mL/min/1.73m²) or markers of kidney damage (albuminuria with albumin to creatinine ratio > 30 mg/g) are present [2]. The progressive decline in kidney function can lead to costly renal replacement therapy that includes dialysis [hemodialysis (HD) and peritoneal dialysis] or kidney transplantation.



				Albuminuria		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g	30-300 mg/g	>300 mg/g
GFR	G1	Normal	≥90			
	G2	Mildly decreasead	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Figure 1 – Risk of chronic kidney disease (CKD) by glomerular filtration rate (GFR) and albuminuria categories**.**

*If no other markers of kidney disease, no CKD; GFR (mL/min/1.73m²). Adapted from KDIGO Group (2012) [2].

1.2. Epidemiology

According to data reported by Jha et al. (2013) [3] the incidence and prevalence of end-stage renal disease (ESRD) (Figure 2) is increasing worldwide and the aging of population contributes to this fact. According to the 2013 Global Burden of Disease Study [4], between 1990 and 2013 the mortality due to CKD increased 36.9% worldwide, with regional differences. The incidence of ESRD in developed countries appears to be slowing, while in developing countries is increasing [5]. In 1990, CKD was ranked 36th in the top 50 causes of global years of life lost (annual age-standardized death rate 11.6 per 100 000), but rose to 19th in 2013 (annual age-standardized death rate 15.8 per 100 000) [4].

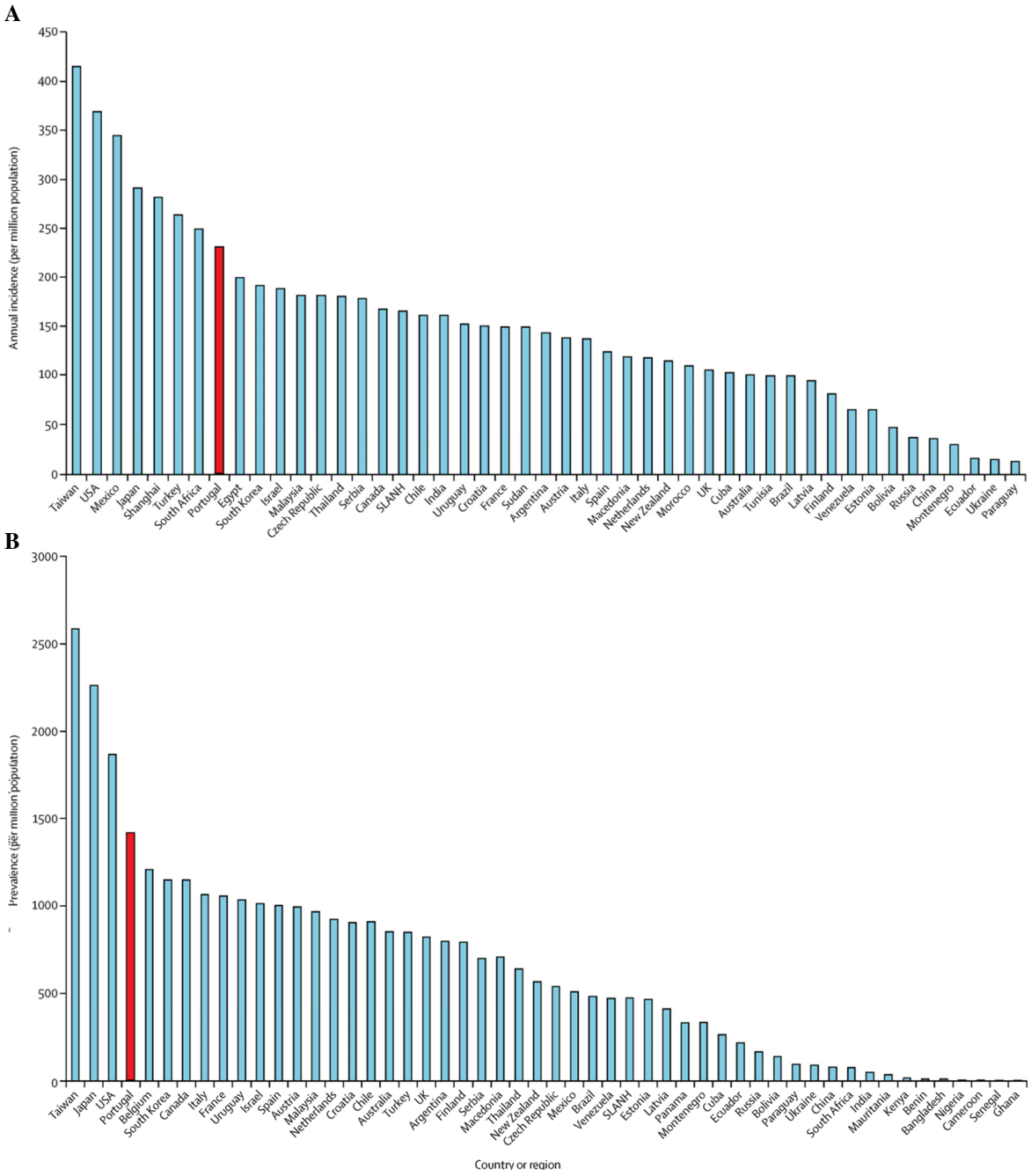


Figure 2 – Incidence (A) and prevalence (B) of end-stage renal disease in different countries.

Adapted from Jha et al. (2013) [3].

In Portugal, the incidence and prevalence of CKD is extremely high when compared to other European countries (Figure 2). The 2013 Global Burden of Disease Study showed that, in Portugal, CKD is in the top 10 causes of potential life lost due to premature mortality and loss of years of productive life due to disability [6]. Data from the "Sociedade Portuguesa de Nefrologia" [7] showed that the incidence (Figure 3) and the prevalence of patients in dialysis therapy increased between 1997 and 2014; in addition, due to increased life expectancy, the majority of incident patients are older than 65 years.

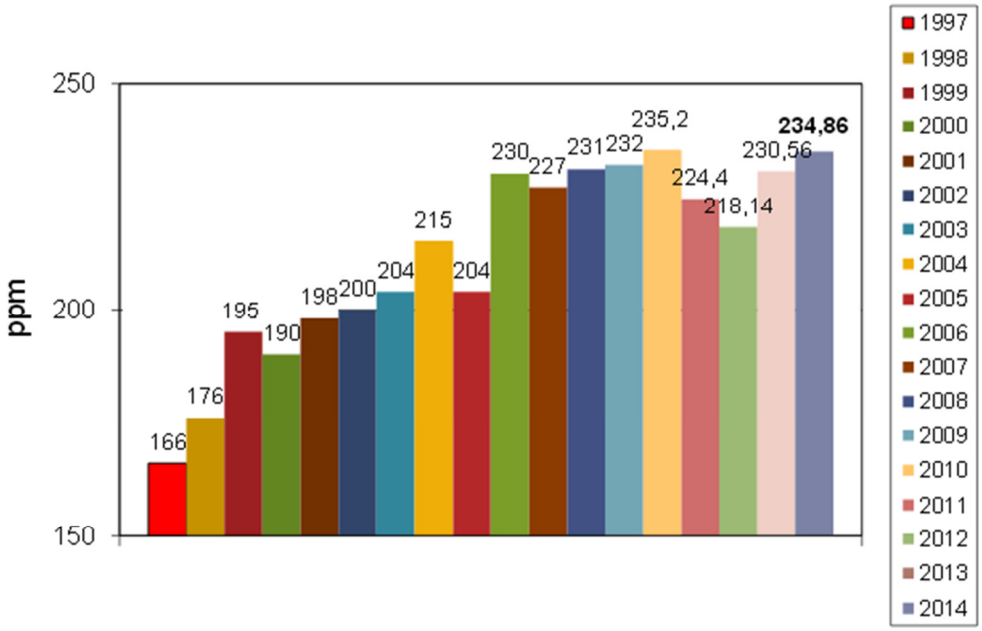


Figure 3 – Annual incidence of dialysis patients in Portugal between 1997 and 2014.
Adapted from "Sociedade Portuguesa de Nefrologia" (2015) [7].

1.3. Etiology and risk factors

According to the location of the pathological-anatomical findings within the kidney, CKD can be classified in several categories (Table 3). CKD can result from primary diseases of the kidneys, such as glomerulonephritis, fibromuscular or renal dysplasia [8]. However, the two main causes of CKD in developed countries are diabetes [mainly type 2 diabetes mellitus] and hypertension [9], as occurs in Portugal (Figure 4), accounting for the majority of deaths in CKD patients [4]. Other causes include inherited kidney diseases, such as polycystic kidney disease, kidney malformations, and acquired kidney diseases, associated with acute kidney injury, recurrent urinary infections, regular use of analgesics (e.g., acetaminophen and ibuprofen) over long periods of time, some nephrotoxic antibiotics (e.g., aminoglycosides), human immunodeficiency virus infection, sickle cell disease, kidney stones and lupus [8-10]. In developing countries, the most common causes of CKD are chronic glomerulonephritis, infections and exposure to drugs and toxins [3, 7].

Several risk factors can contribute to the development and progression of CKD, including age, obesity, African American ancestry, familiar history, and kidney transplant, among others [11-13].

Table 3 – Classification of chronic kidney disease based on the presence or absence of systemic disease and location within the kidney

Diseases	Primary kidney diseases	Systemic diseases affecting the kidney
Glomerular	Glomerulonephritis, glomerulosclerosis, membranous nephropathy	Obesity, metabolic syndrome, diabetes, systemic autoimmune diseases, systemic infections, drugs, neoplasias
Vascular	Fibromuscular dysplasia	Hypertension, atherosclerosis, ischaemia, systemic vasculitis, thrombotic microangiopathy, and systemic sclerosis
Tubulointerstitial	Urinary-tract infections, stones, obstruction	Systemic infections, sarcoidosis, drugs, urate, environmental toxins (eg, lead, aristolochic acid), myeloma
Cystic and congenital	Renal dysplasia, medullary cystic disease, podocytopathies	Autosomal-dominant polycystic kidney disease, Alport syndrome, Fabry disease

Adapted from Eckdart et al. (2013) [9].

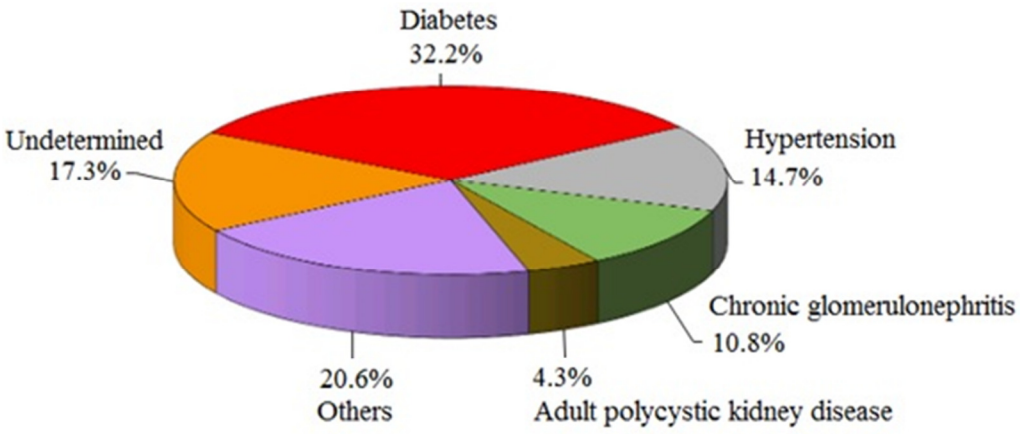


Figure 4 – Etiology of incident dialysis chronic kidney disease patients (n=2446) in Portugal (2014).

Adapted from "Sociedade Portuguesa de Nefrologia" (2015) [7].

1.4. Pathophysiology

CKD can present as the initial cause, glomerular, tubulointerstitial and vascular diseases (Table 3). Irrespective of the initial origin, the histopathological analysis of renal tissue shows that in the final stage of CKD, glomerulosclerosis, tubular atrophy,

tubulointerstitial fibrosis and interstitial inflammatory infiltrate are present in renal biopsies [14-16].

Human studies and the use of animal models have deeply contributed to improve the knowledge on CKD progression [17, 18]. The loss of nephrons (the functional unit of the kidney) is the underlying cause for progressive reduction in renal function. The initial loss of some nephrons is compensated by the remaining nephrons via tissue hypertrophy and hyperfiltration, in order to maintain normal GFR. However, this compensatory mechanism appears to be deleterious for the remaining nephrons, as a progressive deterioration of renal function is observed. Several mechanisms have been proposed to explain the loss of nephrons and progressive CKD (Figure 5).

Glomerular diseases can have many underlying causes as dysregulated, degenerative and inflammatory mechanisms, but all of them will eventually affect podocytes [19]. These cells have major roles in the selective permeability of the glomerular filtration barrier [20] and in the production of vascular endothelial growth factor (VEGF) [21]. The continuous loss or alteration in podocytes will lead to endothelial cell damage, collapse of glomerular capillaries and proteinuria. The glomerular injury is accompanied by a reduction in glomerular blood flow, which leads to a reduction in blood flow into peritubular capillaries, leading to hypoxia and consequent tubulointerstitial injury and renal fibrosis that is considered a final common pathway in CKD [19]. The impact of proteinuria in tubulointerstitial injury and in the progressive renal damage is not well established [22, 23]. In the presence of proteinuria the tubules have to increase reabsorption, with consequent increase in metabolic demands that could lead to hypoxia [24]. The presence of inflammatory cytokines and pro-fibrotic factors in the filtrate may activate tubular cells to produce more of inflammatory and pro-fibrotic factors [25]. In fact, after an initial injury in the tubules, there is an attempt to correct and repair the injury with recruitment and activation of several cells (macrophages, fibroblasts, leukocytes, epithelial tubular cells) that might release cytokines and growth factors. The continuous activation of this system will also lead to excessive extracellular matrix (ECM) accumulation and to increase release of pro-inflammatory cytokines and pro-fibrotic factors, culminating in the formation of scar tissue or fibrosis [25]. The initial stage of tubular injury is characterized by tubular atrophy (loss of brush border and epithelial simplification), associated with diminished transport functions [26]. The progression of tubular atrophy culminates in apoptosis or necrosis of tubular cells [24]. It has been shown that tubulointerstitial lesions, mainly tubular atrophy, correlate better with CKD progression than glomerular lesions [26]. However, due to the higher tubulointerstitial area (about 80%) compared to the glomerular area, a misleading analysis can underestimate the importance of glomerular pathology [26].

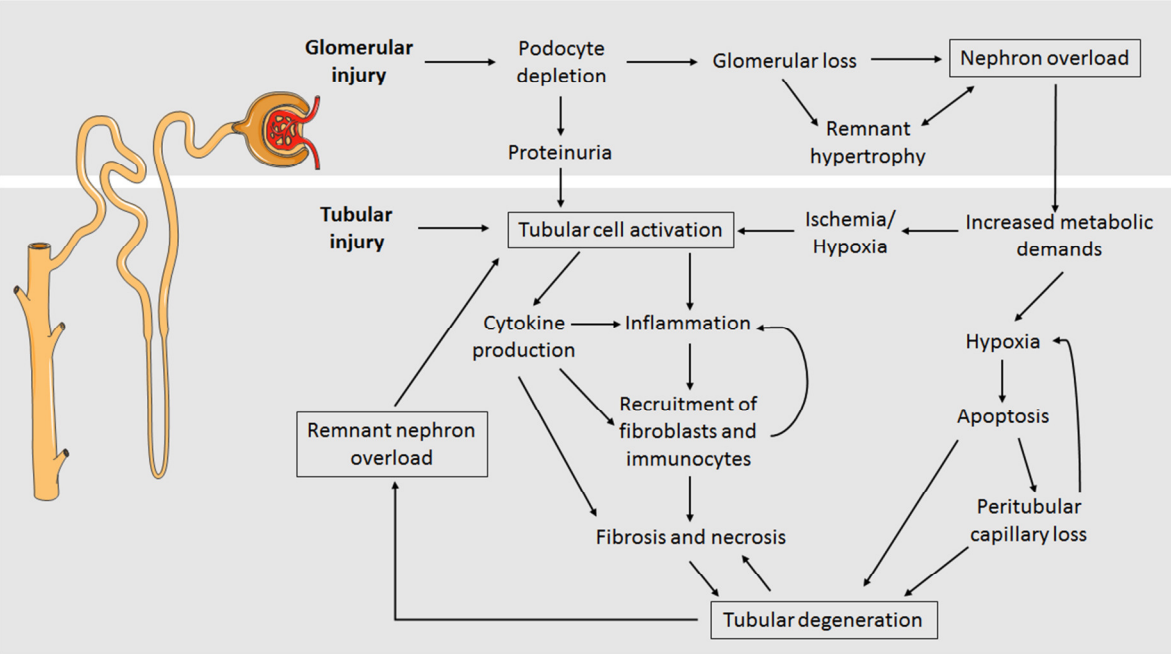


Figure 5 – Schematic diagram of consequences and progression of glomerular and tubular injuries.

Adapted from Hodgkins et al. (2012) [24].

1.5. Complications

The progressive evolution of CKD is associated with several complications, as anemia, worsening hypertension, development and/or progression of cardiovascular disease (CVD), bone disease, hyperkalemia, volume overload, metabolic acidosis, and neurological disturbances, among others.

Hypertension is simultaneously a cause and a complication CKD, and is found in about 80% of CKD patients, increasing its prevalence with decreased renal function [27]. Abnormal sodium homeostasis and increased activity of the renin-angiotensin-aldosterone system (RAAS) are the main causes of hypertension worsening in CKD patients [27].

A systematic analysis for the global burden of diseases, from 1990 to 2013, showed that CVD account for almost a third of all deaths [4], and within CKD patients the mortality rate due to CVD was even higher, especially for ESRD patients [28, 29]. Several risk factors, divided in traditional and non-traditional (Table 4), can contribute to the development and/or progression of CVD in CKD patients.

Many patients with CKD present traditional risk factors (old age, diabetes, hypertension, dyslipidemia and obesity) that, however, do not entirely explain the high CVD rate in CKD patients. Anemia, inflammation, abnormal calcium and phosphate metabolism, increased lipoprotein (a), and malnutrition, among others, are the better known non-traditional risk factors for CVD in CKD patients [10, 30]. The increased risk of

cardiovascular events in patients with CKD seems to result from the conjugation of both traditional and non-traditional risk factors, confirming the very complex pathogenesis of CVD in these patients.

Table 4 – Traditional and non-traditional risk factors for cardiovascular disease in chronic kidney disease patients

Traditional risk factors	Non-traditional risk factors
Age	Anemia
Male gender	RDW
Hypertension	ESA
↑ LDLc	Albuminuria
↓ HDLc	↑ Homocysteine
↓ TG	↑ Lipoprotein(a)
Diabetes	↑Lipoprotein remnants
Smoking	Abnormal calcium/phosphate metabolism
Physical inactivity	Extracellular fluid volume overload
Menopause	Electrolyte imbalance
Family history of CVD	Oxidative stress
Left ventricular hypertrophy	Inflammation (↑ CRP)
Sleep disturbances	Malnutrition
	Thrombogenic factors
	Altered nitric oxide/endothelin balance

Abbreviations: ↑ - increased; ↓ - decreased; apo (a) – Apolipoprotein (a); CRP – C-reactive protein; CVD – Cardiovascular disease; ESA – Erythropoiesis-stimulating agents; HDLc - High density lipoprotein cholesterol; LDLc - Low density lipoprotein cholesterol; RDW - Red cell distribution width ; TG - Triglycerides. Adapted from Weiner (2007) [10], Gargiulo et al. (2015) [30] and Faria et al. (2013)[31].

Mineral and bone disorders are frequent in CKD patients, due to hyperphosphatemia, hypocalcemia, secondary hyperparathyroidism and vitamin D deficiency that can lead to renal osteodystrophy, increasing the risk of bone fractures [32].

Neurological complications, resulting from stroke, white matter diseases, intracerebral microbleeds and cognitive impairment, have increased prevalence in CKD patients, even in mild stages of the disease [33]. The incidence of stroke increases in CKD patients, with a higher incidence in ESRD patients [34]. The increased prevalence of neurological complications may be linked to the traditional and non-traditional risk factors for CVD observed in CKD patients (Table 4).

2. ANEMIA OF CHRONIC KIDNEY DISEASE

2.1. Definition and diagnosis

Anemia is a common complication of CKD that often develops early in the course of the disease, increasing its frequency and severity with the decline of renal function. The incidence of anemia is less than 2% in CKD stages 1 and 2, about 5% in CKD stage 3, 44% in CKD stage 4 and more than 70% in the ESRD [35]. This condition is associated with a decreased quality of life [36, 37], increased hospitalization [38, 39], progression of renal dysfunction [22, 40, 41], cardiovascular complications [42, 43] and mortality [44-46].

The World Health Organization [47] and KDIGO guidelines [48] define anemia as hemoglobin (Hb) concentration below established cut-off levels of less than 12 g/dL for women and less than 13 g/dL for an adult men (Table 5). However, the European Best Practice Guidelines (EBPG) for the management of anemia in patients with CKD recommends that a diagnosis of anemia in these patients should be considered when Hb concentration falls below 11.5 g/dL in women, 13.5 g/dL in adult men and 12.0 g/dL in men older than age 70 [49].

Table 5 – Cut-off levels of hemoglobin concentration to define anemia

Age or gender	Hemoglobin (g/dL)
Children 6 months to 59 months	11.0
Children 5–11 years	11.5
Children 12–14 years	12.0
Non-pregnant women (>15 years)	12.0
Pregnant women	11.0
Men (> 15 years)	13.0

Adapted from World Health Organization (2001) [47].

The diagnosis of CKD anemia should consider the degree and severity of anemia and the type of kidney disease [49]. The parameters that are, usually, used in the clinical assessment of anemia in CKD patients include the complete blood cell count, reticulocyte count, serum ferritin, transferrin saturation (TSAT) and, more recently, the percentage of hypochromic red blood cells (RBC) and reticulocyte Hb content.

The complete blood cell count provides information about the severity and type of anemia, adequacy of nutrients and bone marrow function, and includes the evaluation of Hb concentration, hematocrit (Ht), RBC count, the RBC indices [mean cell volume (MCV), mean cell Hb (MCH) and mean cell Hb concentration (MCHC)], total and differential white blood cell (WBC) count, and platelet count [50]. Usually, the CKD anemia is normocytic (normal MCV: 80-95 fL) and normochromic (normal MCHC: 31.5-34.5 g/dL and MCH: 27-32 pg). However, in the presence of iron deficiency a microcytic anemia develops (low MCV) and, in case of folate or vitamin B12 deficiencies, a macrocytic anemia is observed (high MCV). The WBC and platelet counts helps to distinguish between anemia and pancytopenia (a drop in RBC, granulocytes and platelets), which may reflect ineffective hematopoiesis or bone marrow failure. The anemia associated with hemolysis or hemorrhage usually present neutrophil and platelet counts raised; in case of associated infections the leucocyte count is also often raised [50].

The reticulocyte count (percentage and absolute) and the reticulocyte production index (RPI) are inexpensive and useful to assess the erythropoietic activity. Reticulocytes, the cells released into the circulation that will mature into RBC, are released one day prior to maturation. In a healthy condition, 0.5-2.5% of the circulating RBC are reticulocytes, but in anemia, when the bone marrow is stimulated, more reticulocytes are released into circulation [51]. To face the increased demand in reticulocytes, they are released from the bone marrow in a more immature state, needing, therefore, a longer period in circulation, until they mature into RBC. In case of anemia, the percentage of reticulocytes increases due to a higher production and to a longer period in circulation, needed for their maturation into RBC. Thus, the activity of bone marrow is, more correctly, assessed by calculating the RPI (reticulocyte %/maturation index)*(Ht/normal Ht), as this index corrects for the effect of the premature release of reticulocytes from bone marrow and the severity of the anemia, as worsening of anemia leads to an earlier release of the reticulocytes. In an anemic patient a RPI higher than 3 indicates a normal proliferative response to anemia, whereas a RPI lower than 2 indicates hypoproliferation of bone marrow.

Anemia of CKD is, usually, hypoproliferative with a low erythropoietic activity, due to insufficient renal EPO production. Thus, the EPO deficit leads to a reduced reticulocyte production.

The studies on iron status reflect the level of iron in tissue stores and its availability for erythropoiesis, through the analysis of serum ferritin, TSAT, percentage of hypochromic RBC and reticulocyte Hb content. These tests will be discussed in another chapter (2.2.2 Iron deficiency).

2.2. Etiology

The main cause for anemia in CKD patients is EPO deficit, due to decreased EPO production from the failing kidneys, but other factors can also contribute to the development or worsening of CKD anemia, such as iron deficiency, inflammation, and uremic toxins, among others.

2.2.1. Inadequate erythropoietin production

EPO is a glycoprotein presenting several functions, such as hormone, cytokine and growth factor. EPO acts on target cells that express the EPO receptors (EPOR), through multiple pathways, to control cell proliferation, differentiation and death. EPO is the hormone that promotes erythroid differentiation during erythropoiesis. In the bone marrow, EPO binds to receptors in the early hematopoietic progenitor burst-forming unit-erythroid (BFU-E) cells that differentiate into colony-forming unit-erythroid cells (CFU-E), increasing the EPOR expression, as these cells need EPO for survival [52]. Continued stimulation with EPO triggers differentiation into erythroblasts, which will enucleate to form reticulocytes that mature into RBC after a few days (Figure 6). These two late cell stages (reticulocytes and RBC) do not express EPOR and, therefore, they are not responsive to EPO. The absence of EPO leads to pre-programmed apoptosis of the erythroid cells [52].

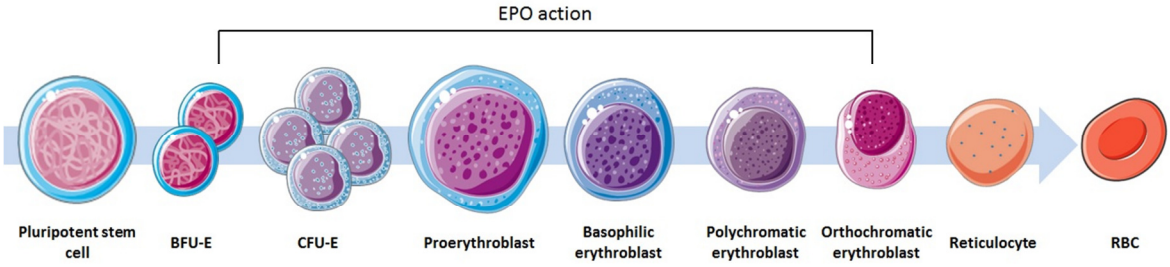


Figure 6 – Erythropoietin in erythropoiesis.

Progenitor cells burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid cells (CFU-E) are responsive to erythropoietin (EPO) stimulation and differentiate in the progenitors of red blood cells (RBC). EPO stimulation continues until progenitors differentiate into erythroblasts. Reticulocytes and mature RBC are no longer responsive to EPO.

During fetal life the majority of EPO is produced by the liver, but after birth there is a switch, and in the adulthood 90% of EPO is produced by the kidney, whereas the liver is a secondary site of production [52]. The kidney cells responsible for EPO production are still a matter of debate, but several studies showed that renal EPO-producing cells (REPC) comprise the peritubular fibroblast-like interstitial cells in the inner cortex and in the outer medulla [53, 54], as well as proximal and distal convoluted tubules and cortical

collecting ducts [55]. REPC are sensitive to changes in oxygen (O_2) tension, and in conditions of hypoxia the kidney responds increasing the number of REPC capable of producing EPO, as tubular cells and renal microvascular endothelial cells, creating a regulatory feedback mechanism [56]. The EPO gene is regulated by hypoxia through a family of heterodimeric transcription factors, known as the hypoxia-inducible factor (HIF) system [56-58]. This HIF system comprises O_2-dependent HIF-1α, HIF-2α (also known as endothelial PAS domain-containing protein 1) and HIF-3α subunits, and the constitutively expressed HIF-1β and HIF-2β subunits (also known as aryl hydrocarbon receptor nuclear translocator). Under normoxia, the HIF-α subunits are hydroxylated, in specific proline residues, by prolyl-4-hydroxylase (PHD) proteins. This functions as a signal for the von Hippel–Lindau tumor suppressor protein (VHL) to work as a substrate for an ubiquitin ligase complement that targets the HIF-α subunits for rapid ubiquitination and proteasomal degradation (Figure 7). However, under hypoxic conditions, as the PHD proteins are inhibited, the HIF-α is not target by VHL protein for degradation and accumulates.

HIF-α subunit translocates to the nucleus, binds to the HIF-β subunit, forming a complex that activates the transcription of several genes (Figure 7), including the EPO gene [56, 57]. Another point of HIF-α control is the hydroxylation of an asparagine residue by the factor-inhibiting-HIF (FIH) in the presence of O_2 that impairs the association of HIF complex with the transcriptional co-activator CBP/p300; however, in hypoxic conditions this hydroxylation is inhibited, as FIH action requires O_2, and the recruitment of CBP/p300 is facilitated, allowing increased levels of transcription (Figure 7) [59].

As EPO is not stored, the mRNA levels reflect the EPO production. Although HIF-1α and HIF-2α share the regulation of many genes, recent studies showed that HIF-2α is the main regulator of EPO synthesis in the kidney [60, 61]. In CKD, the kidney capacity to produce EPO will depend on the severity of the disease [62, 63]. Indeed, it was reported that patients with GFR >30 mL/min/1.73m² retain a physiologic response to anemia, showed by the normal or even elevated serum EPO levels [64, 65]; however, serum EPO levels may not be sufficient for the degree of anemia, as anemic patients with normal renal function may present a 10 to 100 fold increase in serum EPO levels compared to the normal range [66, 67]. In this sense, CKD anemia may be characterized by a relative EPO deficiency rather than an absolute lack.

The kidney is the major site of EPO production in the adults; however, it is possible that in conditions of kidney injury the liver increases its production. In a study using a PHD inhibitor, ESRD patients presented an increase in EPO levels similar to healthy volunteers, suggesting that extrarenal sites can be responsible for a marked rise in plasma EPO [68]. It was also reported that patients with anemia can switch EPO production from

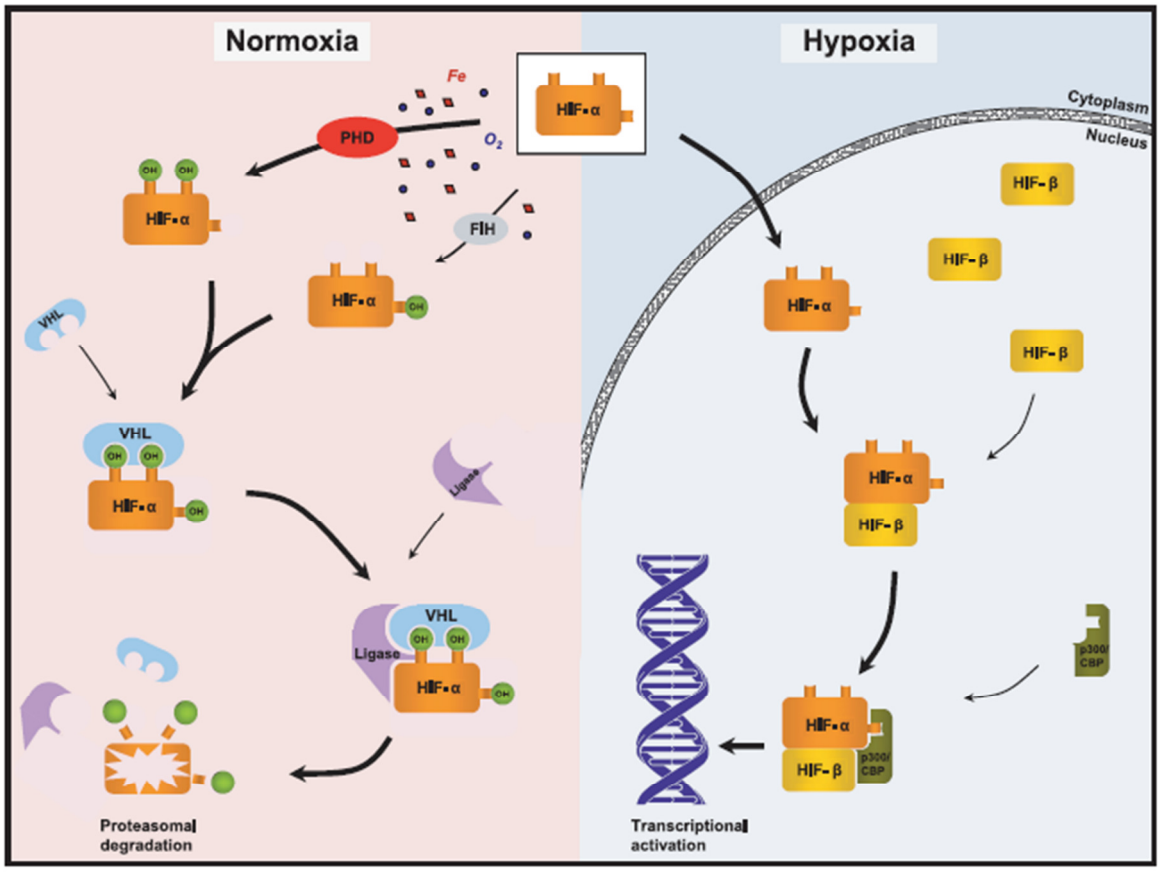


Figure 7 – Schematic representation of hypoxia inducible system.

Proteasomal degradation of hypoxia-inducible factor α (HIF-α) by the von Hippel–Lindau tumor suppressor protein (VHL) requires hydroxylation (OH) by oxygen (O2) and iron (Fe) dependent prolyl-4-hydroxylase (PHD) proteins. Under hypoxia, HIF-α is not degraded and translocates to the nucleus where it forms a heterodimer with HIF-β, activating the transcription of several genes. Adapted from Smith et al. (2008) [69].

the kidney to the liver [70], and that can be showed by glycoform analysis of EPO, as the posttranslational EPO glycosylation is specific to the synthesizing cells, giving rise to different EPO glycoforms, that can be used to localize EPO synthesis [70, 71]. As occurs in the kidney, HIF-2α mediates EPO production in the liver [72, 73]. Hepatocytes, Ito-cells and nonparenchymal cells appear to be the hepatic cells responsible for EPO production, increasing the amount of EPO produced by each cell, in contrast to the kidney that increases REPC number [74-77]. EPO was also found to be expressed in the brain, spleen, lung and testis, although its contribution to serum EPO levels is not clear; EPO expressed in these organs seems to act only at local level [78].

2.2.2. Iron deficiency

Iron is an important element for the erythropoietic process, as it is essential for Hb synthesis. Iron is widely distributed in the organism and is needed for the synthesis of several biological compounds. It is a component of myoglobin, enzymes, cytochromes and

it is important in cellular immune responses. It has been described that iron deficiency leads to a reduced proliferation and function of lymphocytes and natural killer cells and to a diminished neutrophil respiratory burst [79].

The iron in the organism comes from the diet and can be recycled from the internal iron turnover (Figure 8). A normal diet provides 15-20 mg of iron, of which only about 1-2 mg is mostly absorbed in duodenum, through the apical surface of the enterocytes. Dietary iron is provided mainly in the non-heme form (Fe^{3+}) that has to undergo reduction to Fe^{2+}, by the duodenal cytochrome B (DcytB), before enter in the enterocytes, through the divalent metal iron transporter 1 (DMT1). The heme iron present in the diet seems to be promptly absorbed through the membrane protein – heme carrier protein 1 (HCP-1), and released in the enterocyte by the heme-oxigenase-1 (HO-1) [79, 80].

According to the iron requirements, the iron will stay sequestered in the enterocyte in the form of ferritin and lost with the senescence of the enterocyte, or will move to the basolateral membrane, where it is effluxed through the only known iron exporter - ferroportin (FPN). Iron is oxidized by hephaestin, before it binds to plasma transferrin (Tf), the glycoprotein responsible for iron transport to target cells [79, 80]. TfR mediate the cellular uptake of Tf bound iron (Tf-Fe) from plasma. There are two types of TfR, TfR1, which is expressed ubiquitously, and TfR2, mainly present in hepatocytes and erythroid cells, with a lower affinity for Tf-Fe than TfR1 [81].

Tf-Fe is transported to its target cells, such as erythroid, immune and hepatic cells, where it can be used or stored in cytosol, as ferritin, or in the lysosomes, after ferritin breakdown, as hemosiderin [79]. The liver is the major organ for iron storage; however, macrophages can also store iron, as they are responsible for the phagocytosis of senescent erythrocytes in the reticulo-endothelial system [79], and are, actually the main iron storage to be used in erythropoiesis.

The major regulator of body iron homeostasis is hepcidin, a peptide synthesized by the liver that regulates iron absorption and mobilization from stores. The binding of hepcidin to FPN in the membrane of enterocytes, hepatocytes and macrophages, induces its internalization and degradation, thereby, inhibiting iron absorption and mobilization [82]. Hepcidin, the central player of iron metabolism, is synthesized in the liver as a prepropeptide (84 amino acids) that undergoes a cleavage, mediated by furin, producing pro-hepcidin (60 amino acids), which is then processed to produce the mature form – hepcidin (25 amino acids) [83].

Hepcidin can be regulated by several factors (Figure 8). Inflammation, TSAT and liver iron are inducers of hepcidin synthesis, whereas erythropoiesis and hypoxia are inhibitors of hepcidin synthesis.

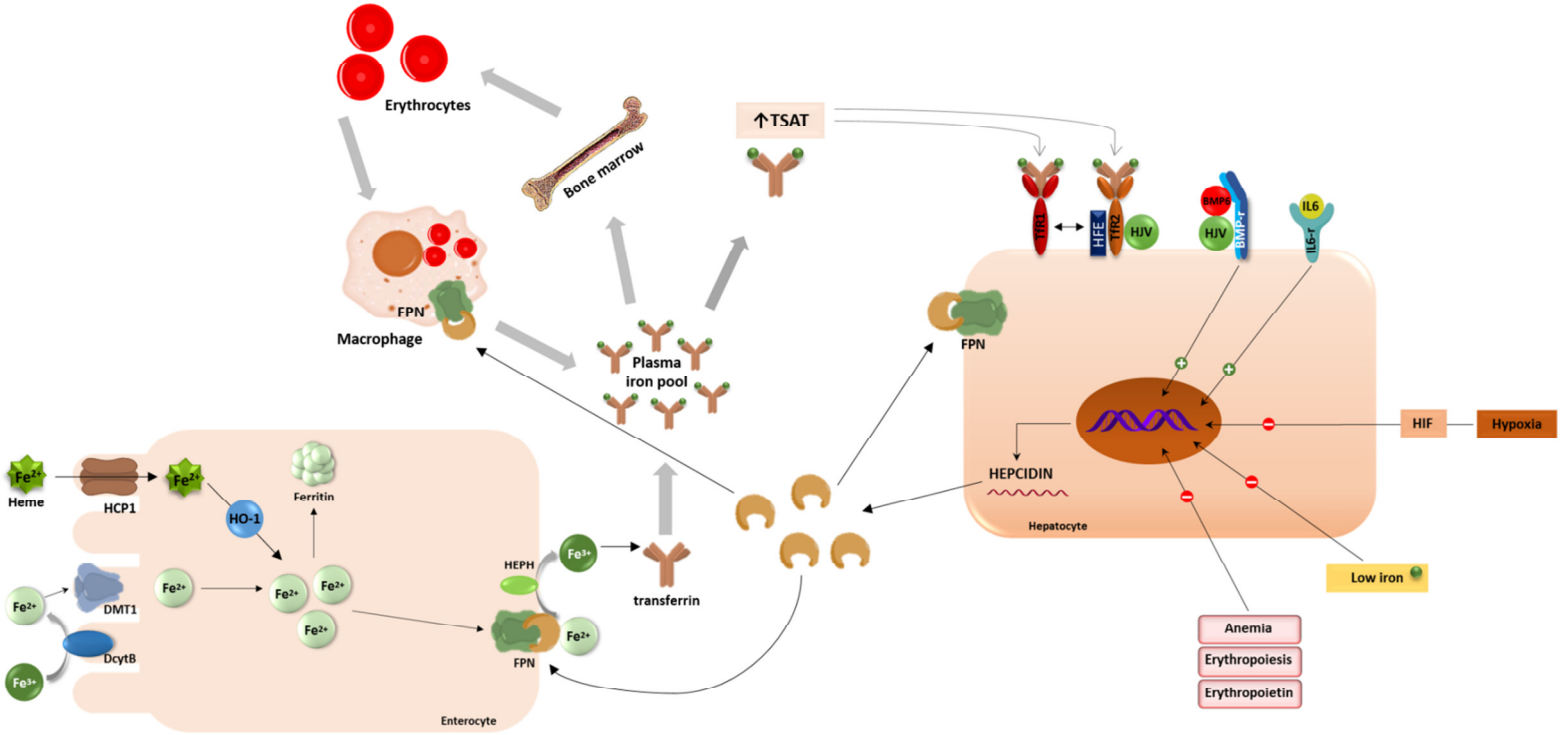


Figure 8 – Iron absorption and regulation of iron metabolism.

Diet iron is present as either heme iron (Fe2+) or non-heme iron (Fe3+). Non-heme iron (Fe^{3+}) must be reduced to Fe^{2+}, by the duodenal cytochrome B (DcytB), to be transported by the divalent metal iron transporter 1 (DMT1). Once inside the enterocyte, the newly absorbed iron enters the intracellular iron pool. If the iron is not required by the body, it is loaded onto the iron storage protein ferritin. Iron required by the body is transferred across the basolateral membrane by ferroportin (FPN). The export of iron also requires the ferroxidase hephaestin (HEPH). Iron is transported by transferrin (Tf) to the local where it is needed, such as into the bone marrow to be used in erythropoiesis. The senescent erythrocytes are phagocyted by macrophages, recycling iron. Hepcidin, the main regulator of iron metabolism (by blocking FPN action), is synthesized by the liver and is regulated by several stimulator (+) and inhibitor (–) factors. Increased transferrin saturation (TSAT) displaces hemochromatosis (HFE) protein from Tf receptor 1 (TfR1) to TfR2, forming a complex that recruits hemojuvelin (HJV), inducing hepcidin synthesis through bone morphogenetic protein 6 (BMP6) and BMP receptor (BMPr). Interleukin (IL)-6 through its receptor (IL6-r) can induce hepcidin synthesis. Anemia, increased erythropoiesis, erythropoietin and hypoxia (probably through hypoxia inducible factor [HIF]) are factors responsible for hepcidin inhibition. Adapted from Ribeiro et al. (2015) [84].

Several tests can be used to assess iron deficiency. Plasma/serum ferritin concentration is the only blood marker to assess iron stores; a value lower than 30 ng/mL in men or 15 ng/mL in women is consistent with absolute iron deficiency. On the other hand, ferritin levels higher than 300 ng/mL, along with anemia, indicate a functional iron deficiency, as frequently occurs in CKD patients [49]. TSAT is a measure of circulating iron, available for delivery into the bone marrow; a value lower than 20% indicates iron deficiency [49]. It is important to distinguish between absolute and functional iron deficiency. Low levels of iron, ferritin and TSAT reflect absolute iron deficiency, which is responsive to iron therapy. A functional iron deficiency presents with high levels of ferritin, low iron and low/normal levels of TSAT that, usually, results from an inflammatory state. C-reactive protein (CRP) is widely used to assess inflammation, and present concentrations higher than 3 mg/L in inflammatory conditions [49]. Due to the limitations of ferritin and TSAT analysis, as they are influenced by inflammation and general nutritional status [85], other parameters that are included in the EBPG guidelines can also be useful to assess iron status [49]. The value of serum soluble TfR (sTfR) reflects the number of erythroblasts in the bone marrow and/or the need for iron. Indeed, under conditions of iron deficiency, the number of erythroblasts will increase in the bone marrow, as well as the number of membrane TfR in the erythroblasts. Some of the TfR are released along cell maturation into the circulation, where they can be evaluated. Higher levels of sTfR usually indicate iron deficiency or deficient iron mobilization; in the anemia of chronic disease or anemia of inflammation, the levels of sTfR are within normal range or high. This parameter is very useful as it is less affected by inflammation. The availability of iron for erythropoiesis can also be assessed by the percentage of hypochromic RBC and reticulocyte Hb content. The percentage of hypochromic RBC increases in case of absolute or functional iron deficiency [86], and is affected by erythropoietic activity [87]. The reticulocyte Hb content, measuring the amount of Hb in reticulocytes, increases in the case of absolute or functional iron deficiency [85], and is influenced by MCV [88].

Iron deficiency is a common factor for CKD anemia, mainly in HD patients, that lose about 1–2 g of iron per year. The high frequency of blood analysis, the surgical procedures for vascular access, the blood loss into the hemodialyser (and tubes) during the dialysis procedure, and the presence of inflammation contribute to iron deficiency. Moreover, in CKD patients, iron homeostasis may be disturbed at several points of its cycle, including absorption, release from its storages and utilization. For instance, high hepcidin levels that are common in CKD patients [31, 89], can contribute to iron deficiency, due to its effect on iron absorption and mobilization from stores. Inflammation, also common in CKD patients [31, 90], is one of the major contributors for functional iron deficiency, due to its stimulatory effect on hepcidin synthesis (Figure 9A). Hepcidin was first reported by its

antifungal and antimicrobial properties [91] and was considered an acute-phase protein [92]. The best characterized cytokine responsible for inducing hepcidin synthesis is interleukin-6 (IL-6) [93], although other cytokines have been later reported as hepcidin synthesis stimulators [94, 95]. IL-6 mediated hepcidin activation occurs through the activation of the signal transducer and activator of transcription 3 (STAT3) pathway [96, 97]. Recently, it was reported that activin B, also increased in inflammatory states, can induce hepcidin synthesis through the son of mothers against decapentaplegic (SMAD) 1/5/9 pathway, independently of IL-6 pathway [98].

Iron is a strong inducer of hepcidin synthesis (Figure 9B). TSAT is a sensor for circulating iron and, therefore, controls iron systemic homeostasis. In normal serum iron levels (normal TSAT), diferric Tf competes with hemochromatosis (HFE) protein for binding to TfR1; in conditions associated with increased iron (increased TSAT), more HFE will be available to bind to TfR2; this complex, TfR2-HFE, recruits hemojuvelin (HJV) an important co-factor for bone morphogenetic protein 6 (BMP6) [99, 100]. The BMP6-HJV complex activates the BMP receptor that will induce the phosphorylation of SMAD1/5/9 proteins, which recruits SMAD4, forming a complex that translocates to the nucleus, triggering hepcidin synthesis [101]. It was suggested that the complex TfR2-HFE can also induce hepcidin synthesis through the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway and furin [102], but this pathway was not proved to occur *in vivo* [103].

Other proteins are involved in the fine-tuning of hepcidin expression. Neogenin appears to present two distinct functions, as it acts as a HJV stabilizer, enhancing BMP signaling [104, 105], and interacts with matriptase-2 to promote HJV cleavage [105]. Matriptase-2 is a recognized negative modulator of hepcidin synthesis; due to its activity as a membrane serine protease, it cleaves HJV that is released in a soluble form, blunting the BMP pathway [106, 107]. The regulation of matriptase-2 is still under study but several stabilizers and inducers have been reported, such as low intracellular iron [108], HIF system [109], and (indirectly) circulating iron and BMP6 to control iron overload [110]. Hepatocyte growth factor activator inhibitor type 2 [111] and inflammation (through STAT5) [112] modulates matriptase-2 inhibition. SMAD7 emerged as an inhibitor of hepcidin synthesis by its interaction with SMAD4, thus blunting the SMAD pathway activation [113]. Recently, endofin responsible for the recruitment of the SMAD proteins to BMP receptors was identified as another protein involved in the regulation of hepcidin synthesis [114].

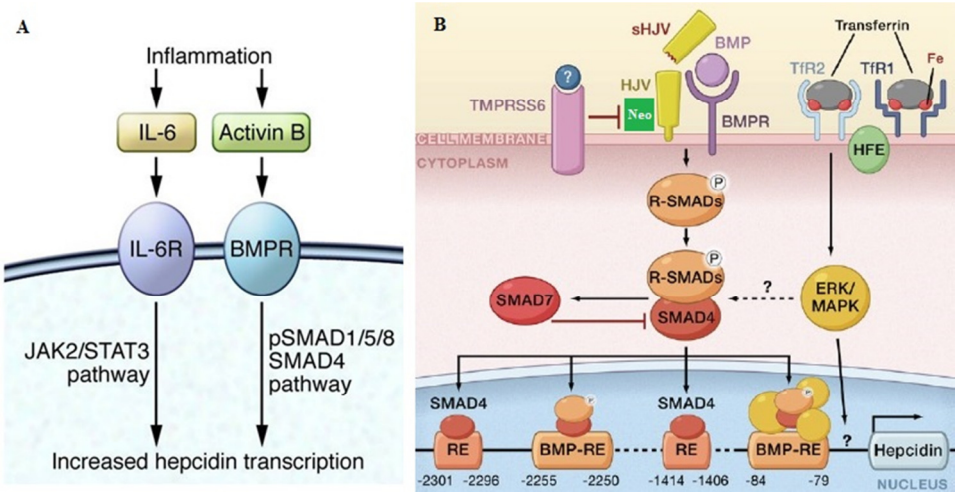


Figure 9 – Regulation of hepcidin expression.

A) Regulation of hepcidin by inflammation. Interleukin-6 (IL-6) activates the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, whereas activin B activates the son of mothers against decapentaplegic (SMAD) pathway, both inducing hepcidin synthesis. B) Regulation of hepcidin by transferrin. Increased transferrin saturation displaces hemochromatosis (HFE) protein from transferrin receptor 1 (TfR1) to TfR2, forming a complex that recruits hemojuvelin (HJV) and neogenin (Neo), activating the bone morphogenetic (BMP)-SMAD pathway inducing hepcidin transcription. The TfR2-HFE complex can possibly also act through the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway. Matriptase-2 (TMPRSS6) promotes cleavage of HJV, releasing a soluble form (sHJV) that blunts the BMP-SMAD pathway. SMAD7 inhibits the SMAD pathway by interacting with SAMD4. Adapted from Zhao et al. (2013) [115] and Hentze et al. (2010) [116].

Liver iron stores are also modulators of hepcidin synthesis, responsible for cellular iron homeostasis [117]. Liver iron appears to induce directly BMP6 expression and activation of the SMAD1/5/9 pathway [103]; however, the mechanism by which hepatic iron levels regulate BMP6 remains to be elucidated.

When blood O_2 saturation decreases and hypoxia occurs, erythropoiesis is stimulated and the iron demand for erythropoiesis in the bone marrow increases. Thus, it is necessary to increase iron absorption and the mobilization of iron stores. Both erythropoiesis and hypoxia down-regulate hepcidin expression [118, 119]. Although, intense studies have been made in this field, the exact mechanisms by which erythropoiesis and hypoxia inhibit hepcidin are poorly understood.

Enhanced erythropoiesis is accompanied by an increase in serum EPO levels and bone marrow erythropoietic activity. It was reported that EPO can directly suppress hepcidin synthesis [120], but several studies *in vivo* showed that EPO inhibition of hepcidin occurs indirectly by erythropoiesis [121-124]. The most accepted mechanism is that bone marrow releases factors produced during erythropoiesis that control hepcidin synthesis. Several candidates have been proposed, as growth differentiation factor 15 [125, 126], twisted gastrulation 1 [127] and, more recently, erythroferrone [128, 129]; however,

the exact factor(s) and mechanism(s) is still unclear. Increased erythropoietic activity, as occurs in response to anemia, is accompanied by HIF system stimulation, due to hypoxia. The effect of the HIF system on hepcidin synthesis also appears to occur indirectly via erythropoiesis, but no consensus is still available [130-132]. It was also reported that, in hepatocytes, hypoxia inhibits hepcidin synthesis by reducing SMAD4 protein [133] and, as already referred, matriptase-2 (hepcidin negative modulator) can be regulated by the HIF system [109, 134]. Furin, that is responsible for hepcidin cleavage, also appears to act on HJV in a HIF-dependent manner [135]. In addition, the HIF system is also responsible for the expression of several fundamental elements in iron cycling, such as Tf, TfR1, hephaestin, DMT1, DcytB and HO-1 [136-138].

2.2.3. Inflammation

Inflammation modulates not only hepcidin synthesis, contributing to functional iron deficiency, but also affect EPO synthesis and erythropoiesis [51]. Inflammation starts early in the progress of renal disease [139] and is often observed in HD patients [27, 90]. The etiology of inflammation in these patients is multifactorial and includes intercurrent clinical events, comorbidities, renal disease and dialysis [140, 141]. The inflammatory state can be evidenced by the rise in several pro-inflammatory markers, such as IL-6, IL-1β, tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ), and by the rise in the acute phase reactant CRP.

Inflammation contributes to CKD anemia through several ways. It can directly suppress erythropoiesis, due to the inhibitory effect of IL-1β, TNF-α and IFN-γ on BFU-E and CFU-E stages in bone marrow [142-145]. IL-6 can impair erythroid production by inducing mitochondrial dysfunction in maturing erythroid cells, which leads to impaired Hb synthesis and erythroid maturation [146]. The bone marrow macrophages can be stimulated to increase local pro-inflammatory cytokines, amplifying the effects of systemic inflammation [147]. Inflammation can also influence EPO production [148-150]. It was recently reported that HIF system can also be affected by inflammation, through the induction of the aryl hydrocarbon receptor that competes with HIF-2α for binding with HIF-β [151]. Reduced RBC lifespan is also a consequence of inflammation due to increased phosphatidylserine exposure, increased phosphorylation of membrane protein Band 3 and reduced deformability, all markers for RBC phagocytosis by macrophages [152].

2.2.4. Uremic toxins

In CKD, the progressive loss of glomerular filtration capacity of potentially toxic compounds leads to the accumulation of uremic solutes in the blood stream that are known as uremic toxins, biologically or biochemically active. The European Uremic Toxin

(EUTox) Work Group [153] listed more than 100 different uremic retention solutes, classified in three major groups (Table 6), according to their physical-chemical characteristics that may influence solute removal by dialysis or related strategies [154].

Uremic toxins can induce anemia by reducing the RBC lifespan or by causing hypoproliferation of red cell progenitors in the bone marrow, through an inhibitory effect.

Uremia associated to inflammation and oxidative stress are responsible for an acceleration of functional changes and disruption of the RBC membrane [155]. This can lead to a mechanism known as eryptosis (suicidal death of erythrocytes), whose features are cell shrinkage, membrane blebbing and exposure of phosphatidylserine, a signal for recognition by macrophages and subsequent elimination of these RBC from circulation [152]. It has been reported that uremic toxins increase O_2 consumption and aggravate local hypoxia in renal tubular cells through a mechanism involving the development of oxidative stress, triggering disturbances in the O_2 sensing system and in EPO production [156].

Table 6 – Classification of uremic retention solutes

Classification	Characteristics	Examples
Small water-soluble molecules	MW <500 Da easily removed by any dialysis strategy	Urea, creatinine
Middle molecules	MW >500 Da only removed through large-pored membranes	β2-M, TNF-α, IL-1β, IL-6
Protein-bound molecules	Any MW difficult to remove with any dialysis strategy	Homocysteine, indoles

Abbreviations: β2-M - β2-Microglobulin; Da – Daltons; IL- Interleukin; MW – Molecular weight; TNF – Tumor necrosis factor. Adapted from Vanholder et al. (2003) [154].

2.2.5. Others

Chronic blood loss occurs is dialysis patients, especially in those on HD, due to uremic platelet dysfunction [157], blood retained and lost within the dialysis circuit, blood drawn for laboratory monitoring studies, and to bleeding events [49]. Blood loss results in iron loss that may lead to depletion of iron and development of iron deficient anemia.

Malnutrition, aluminum accumulation, deficiency in folic acid or vitamin B12, and some drugs, such as angiotensin-converting-enzyme (ACE) inhibitors, can also contribute to anemia in CKD patients [49].

2.3. Anemia and progression of renal disease

Anemia is associated with the progression of renal dysfunction [22, 40, 41] and the use of animal models have been helpful to elucidate the underlying mechanisms. As above mentioned, the main cause of anemia is the inadequate EPO production by the kidneys. It is proposed that after an injury REPC can suffer a transdifferentiation, called epithelial to mesenchymal transition (EMT), into myofibroblasts, thus, losing their capacity to synthesise EPO [158]. The increase in myofibroblasts, responsible for collagen synthesis, leads to excessive ECM deposition. These cells seem to derive from several cells, as fibroblasts, pericytes, tubular epithelial cells (such as endothelial cells), and bone marrow derived cells (fibrocytes and mesenchymal stem cells), although its origin is still debatable [53, 159, 160]. Transforming growth factor beta (TGF-β), a recognized pro-fibrotic factor, appears to be central for fibroblast activation, proliferation and transdifferentiation, contributing to ECM deposition [161]. TGF-β presents immunomodulatory effects on macrophages and monocyte recruitment, leading to the production of inflammatory cytokines [162]. Initially, in renal injuries, M2-type macrophages are recruited to promote tissue remodelling; however, if this process is continuous more inflammatory monocytes are recruited that will differentiate into M1-type macrophages, which produce pro-inflammatory cytokines (such as TNF-α, IFN-γ, IL-1β and IL-6) and induce apoptosis [163]. The release of these pro-inflammatory cytokines leads to the activation of the nuclear factor kappa B (NF-κB) pathway, thus, amplifying the inflammatory process [162]. The initial response occurs as an attempt to reduce/overcome renal injury. When not achieved, the continuous activation and excessive ECM accumulation contribute to the formation of scar tissue and renal fibrosis, creating a vicious cycle that will lead to decreasing EPO production, development of anemia and tissue hypoxia.

Tissue hypoxia is amplified according to the severity of anemia that will reduce O_2 availability to the organs. Within the kidney, the hypoxic environment leads to the activation of the HIF system, promoting the transcription of several target genes. Although HIF-1α and HIF-2α share the activation of several genes, HIF-2α is the main regulator of EPO gene, whereas HIF-1α is responsible for regulating glycolytic enzyme genes, as well as the genes involved in renal fibrosis [164]. Renal biopsies from CKD patients showed increased expression of HIF-1α in tubular epithelial cells that correlates to the stage of renal disease [164]. Connective tissue growth factor (CTGF) is directly regulated by HIF-1α and is a potent pro-fibrotic factor, as it potentiates TGF-β signaling [164] and appears to promote EMT [165]. HIF-1α was also associated to the EMT process [166]. It was reported that in CKD HIF-α activation presents dynamic changes, being activated in early CKD stages and suppressed in the middle and end-stage of CKD [167]. In a study using a PHD inhibitor, the early activation of the HIF system, triggering mainly

the up-regulation of the HIF-1α subunit, was associated with renal fibrosis and exacerbated renal dysfunction; the activation of the HIF system by the PHD inhibitor in a more advanced stage, where HIF-2α was also up-regulated induced VEGF and EPO production [63]. Inflammation and hypoxia are closely linked as tissue hypoxia induces inflammation, and vice-versa, through a NF-κB-dependent pathway that induces HIF-1α up-regulation [168]. The initial up-regulation of HIF-1α promotes injury repair, but the continuous activation contributes to worsening of tissue hypoxia and progression of renal disease [169].

2.4. Treatment

The correction of anemia should be based on the diagnosis and identification of correctable causes [48]. In patients with a known inflammatory state (including bacterial and viral infections) the reduction of inflammation can be associated with Hb increase [48].

The use of RBC transfusion is an option for CKD chronic anemia correction [48], but the risk of transfusion reactions (immunological sensitization), transmission of infectious agents and iron overload are limitations for the use of this treatment [170].

The standard treatment for CKD anemia is based on pharmaceutical intervention by the use of erythropoiesis-stimulating agents (ESA) and/or iron supplementation, in order to maintain Hb concentration in the range of 10-11.5 g/dL [48]. ESA therapy will be discussed in the next chapter. The current guidelines refer that CKD patients with absolute iron deficiency should start a trial using oral supplementation, when TSAT is lower than 30% and ferritin levels lower than 500 ng/mL. In case of functional iron deficiency, normal TSAT and ferritin higher than 300 ng/mL, iron supplementation is also recommended, in order to maintain iron available for an adequate erythropoiesis; however, the upper safe serum ferritin level was not defined. The decision for oral or intravenous (IV) iron treatment should balance the benefits and risks for the patient (Figure 10).

Oral iron formulations (Table 7) are less expensive; however, the gastrointestinal side effects experienced by some patients (about 30%) may reduce the effect and the adherence to treatment [171, 172]. Some studies in anemic non-dialysis (ND)-CKD patients reported that IV iron therapy increases both Hb and ferritin, while oral iron therapy increases Hb without increasing iron stores [171, 172]. Moreover, most of the clinical studies evaluating oral and IV administration reported that IV iron therapy leads to a higher increase in Hb than oral iron therapy [172, 173].

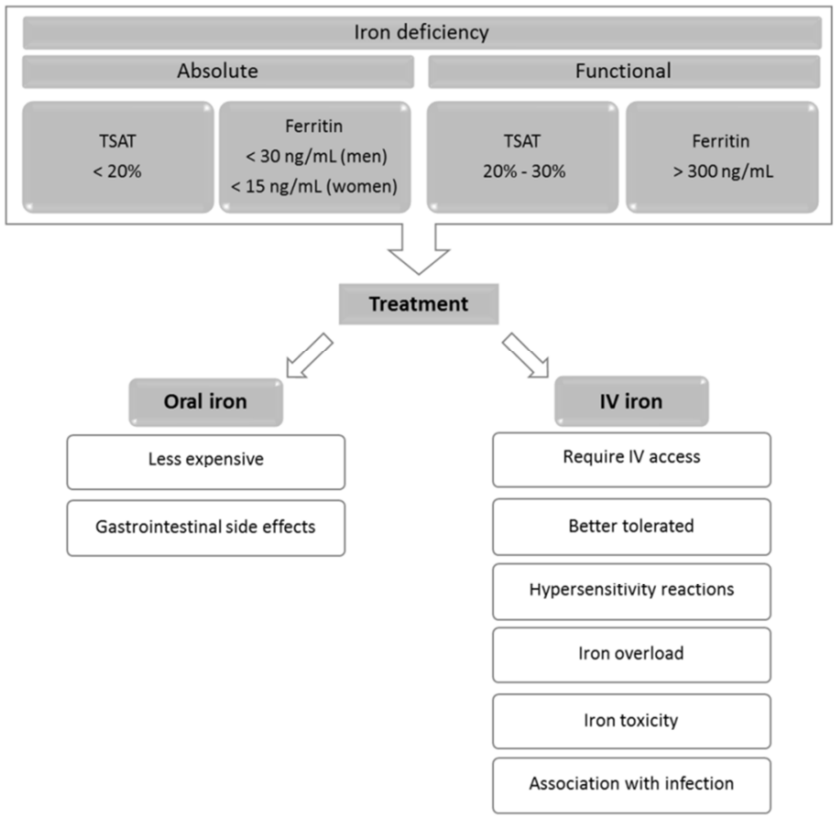


Figure 10 – Iron deficiency and treatment of anemia in chronic kidney disease patients. Iron deficiency can be absolute or functional according to transferrin saturation (TSAT) and ferritin values. Oral or intravenous (IV) iron can be used to correct iron deficiency depending on patient characteristics. Adapted from Ribeiro et al. (2015) [84].

Table 7 - Iron supplements approved for chronic kidney disease anemia treatment

Oral	**Intravenous**
Ferrous sulfate	Iron sucrose
Ferrous gluconate	Ferumoxytol
Ferrous fumarate	Ferric carboxymaltose
	Iron dextran

Adapted from Ribeiro et al. (2015) [84].

IV iron therapy is better tolerated than oral iron therapy [171, 172], has more adherence and efficacy, but requires the existence of an IV access. It has been associated with hypersensitivity reactions that, although very rare, can be life-threatening [174]. The major risk factors for these hypersensitivity reactions include a previous reaction to an

iron infusion, a fast iron infusion rate, multiple drug allergies, severe atopy and systemic inflammatory diseases [174].

IV iron therapy is preferred for patients under HD, as they have a vascular access already available that can be used for iron infusion. In ND-CKD patients, oral iron may be preferred to preserve IV accesses. However, there is no consensus for the use of oral rather than IV iron as first line treatment in ND-CKD patients. Several studies have been performed [171, 173, 175, 176] to further clarify the efficacy and safety of oral *versus* IV iron supplementation in ND-CKD patients. Stoves et al. (2001) [175] in a follow-up study along 6 months of ND-CKD patients, found that the Hb response and ESA requirements with IV iron (iron sucrose) or with oral iron (ferrous sulfate) supplementation were similar. In accordance, Charytan et al. (2005) [171] and Agarwal et al. (2006) [177] reported that both IV and oral iron therapies in ND-CKD patients resulted in similar Hb responses, but IV iron therapy showed better results in replacing iron stores. Conversely, Qunibi et al. (2011) [172], Van Wyck et al. (2005) [173] and the recently FIND-CKD study (2014) [176] found that IV iron is superior to oral iron therapy, as ND-CKD patients under IV iron therapy showed higher Hb levels and iron stores, as well as a higher improvement in their quality of life.

The efficacy of oral iron therapy can be compromised in CKD, as these patients usually present a low to mild degree of inflammation, which leads to an increase in hepcidin levels [31, 89]. This increase in hepcidin, by reducing gastrointestinal iron absorption and decreasing iron release from body storage sites, inhibits the use of iron for erythropoiesis and leads to an increase in ferritin, also functioning as an acute phase protein.

The optimization of iron delivery, given the complications associated with IV iron therapy, and the reduced oral iron adherence and efficacy, is a current challenge to search and develop better iron formulations (Table 7).

3. ERYTHROPOIESIS-STIMULATING AGENTS

3.1. Pharmacology

Purification of human EPO from the urine of patients with aplastic anemia [178] allowed its characterization, and the isolation and cloning of the human genomic DNA encoding for EPO [179]. EPO is a glycoprotein of 166 amino acids with a molecular weight (MW) of 34 kDa and 30% carbohydrate, containing sialic acid and N-acetylglucosamine. The glycosylation of EPO is important for its *in vivo* activity and stability [180].

After cloning the EPO gene, the modern technology of DNA recombination allowed the production of recombinant human erythropoietin (rHuEPO). rHuEPO synthesized by (Chinese) hamster ovary cell lines is highly glycosylated, but the carbohydrate structure is similar to human EPO. rHuEPO presents a MW of 30 kDa and contains approximately 39% carbohydrate [181], but this does not affect its activity [182].

Epoetin beta and epoetin alpha were the first rHuEPO approved for anemia treatment (Table 8). These isoforms present some differences, but with no effect on its clinical efficacy [183].

The pattern of glycosylation affects the half-life of rHuEPO; based on that, other ESA appeared, with similar actions, but differing in their half-life, which determines the frequency and route of administration (usually, the IV administration is more convenient for HD patients) leading to the division in “short-acting” and “long-acting” ESA (Table 8).

Darbepoetin alpha presents two additional N-linked carbohydrate chains compared to EPO, which increase its half-life, allowing its administration only once per week [184]. In 2007, methoxy polyethylene glycol-epoetin beta, a continuous EPO receptor activator was introduced, presenting a half-life of about 130 h, which reduced its administration for once per month [185]. Peginesatide, with a longer half-life, is a pegylated homodimeric peptide with no sequence homology to EPO [186]; it was the first synthetic peptide approved by the Food and Drug Administration (FDA) for the treatment of CKD anemia, in 2012; however, due to serious hypersensitivity associated reactions, the product was removed from the market in 2013. Due to expire of several patents, ESA biosimilars (Table 8) have been approved by the European Medicines Agency (EMA), presenting therapeutic profiles similar to those of epoetin beta and alpha [184].

The need for better formulations and other strategies for drug delivery are growing fields in the treatment of anemia (Table 9). EPO fusions proteins - Fc-EPO, CTNO 528 – can be administered as an aerosol [187], and other delivery systems are under investigation, such as ultrasound-mediated transdermal uptake and via liposomes (orally) [188]. The HIF stabilizers have the advantage that can be administered orally. Several HIF

Table 8 – Approved erythropoiesis-stimulating agents

ESA	Approval		Structure	Half-life	Freq. adm.	Therapeutic area
	FDA	EMA§				
Short-acting						
Epoetin beta		1989	Identical aa and carbohydrate composition to EPO	IV 4-12h SC 12-28h	3 x/w	Anemia, CKD, cancer, blood transfusions
Epoetin alpha	1989	1989		IV ≈5h SC ≈24h	3 x/w	Anemia, CKD Cancer
Epoetin zeta*		2007		IV ≈5h SC ≈24h	3 x/w	Anemia, CKD, cancer, blood transfusions
Epoetin theta*		2009		IV ≈4h SC ≈34h	3 x/w	Anemia, CKD Cancer
Long-acting						
Darbepoetin alpha	2001	2001	2 additional N-linked carbohydrate chains compared to EPO	IV ≈21h SC ≈73h	1 x/w	Anemia, CKD Cancer
Methoxy polyethylene glycol-epoetin beta	2007	2007	Continuous EPO receptor activator	IV ≈134h SC ≈139h	1 x/m	Anemia, CKD

*Biosimilars; §From 1995 to 2009, the European Medicines Agency was known as European Agency for the Evaluation of Medicinal (EMEA) Products. Abbreviations: aa – amino acid; CKD – Chronic kidney disease; EMA – European Medicines Agency; EPO – Erythropoietin; ESA – Erythropoiesis-stimulating agents; FDA – Food and Drug Administration; Freq. adm. – Frequency of administration; IV – Intravenous; m – month; SC – Subcutaneous; w – week. Adapted from Ribeiro et al. (2013) [189].

stabilizers are under clinical trials, mainly PHD inhibitors [190]. However, there are some concerns about the tumorigenicity of these agents [191], as they present the disadvantage of up-regulate other HIF-sensitive genes, besides the EPO gene. Therefore, the current attempt is to find agents able to up-regulate only erythropoiesis genes. The most attractive area on this field is the EPO gene therapy, which could allow the generation of lower and continuous levels of EPO [192]. Several strategies have been studied [193, 194], but their application in humans needs more studies to evaluate the safety profile of these agents.

Table 9 – Erythropoiesis agents under study

Protein-based ESA therapy	EPO fusion proteins
Small-molecule ESA	Peptide-based Non-peptide based
Other strategies	HIF stabilizers GATA inhibitors HCP inhibitors EPO gene therapy

Abbreviations: EPO – Erythropoietin; HIF – Hypoxia inducible factors; HCP – Hemopoietic cell phospathase. Adapted from Ribeiro et al. (2013) [195]

In healthy humans, rHuEPO treatment increases Hb concentration and, thus, arterial oxygen content, by depressing plasma volume and increasing RBC mass [196]. The reduction of plasma volume may result from the reduced reabsorption of water and sodium in the proximal renal tubules promoted by rHuEPO and, probably, through a mechanism involving the reduction of the RAAS activity [197].

The mechanisms for ESA clearance are not well elucidated, but several pathways can be involved. Clearance by hepatic and kidney pathways are considered [198], but the presence of liver or kidney disease appears to unaffected ESA clearance [199, 200], suggesting the presence of other mechanism, as receptor-mediated endocytosis of EPO via EPOR followed by degradation in lysosomes, with a major role for the bone-marrow [201, 202].

EPO and ESA stimulate erythropoiesis by binding to EPOR homodimers, present in the membrane of erythroid progenitors. EPO binding triggers a conformational change that stimulates auto-phosphorylation of janus kinase 2 (JAK2), which phosphorylates tyrosine residues on the EPOR intracellular domains, activating cytoplasmic signaling proteins, such as STAT5, AKT and ERK1/2 pathways, responsible for cellular differentiation and proliferation and for anti-apoptotic effects (Figure 11) [58].

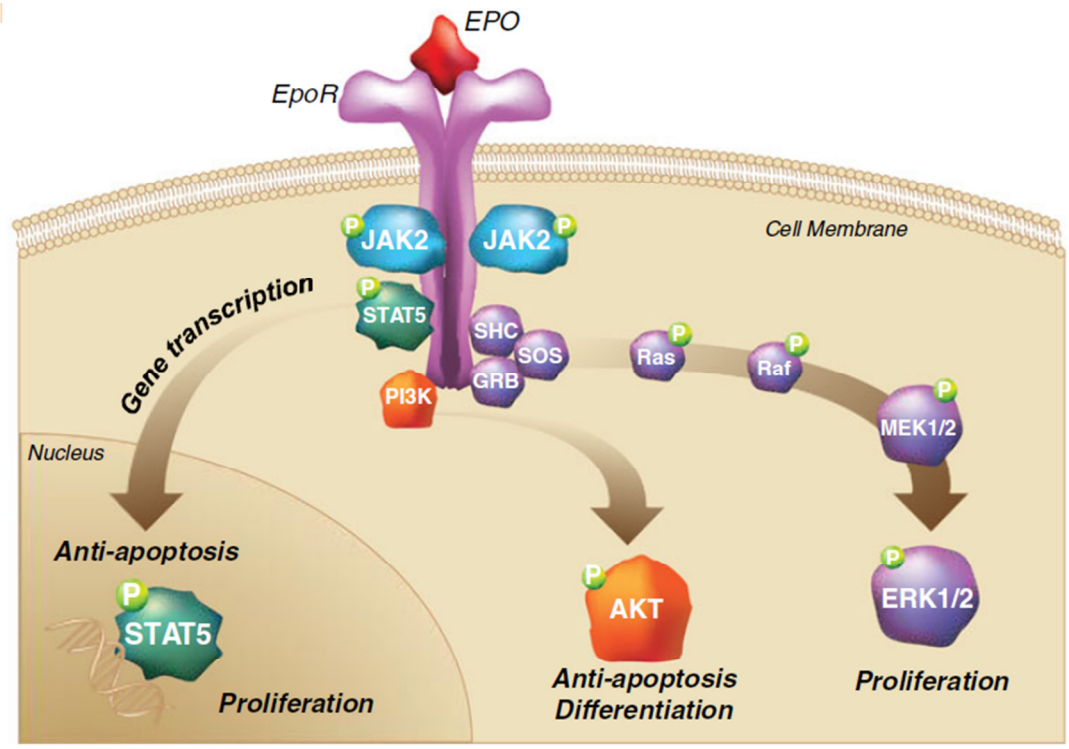


Figure 11 – Signaling pathways stimulated by the linkage of erythropoietin to erythropoietin receptor.

Erythropoietin (EPO) and erythropoiesis-stimulating agents binding to EPO receptor (EPOR) triggers the activation of several signaling pathways, such as the signal transducer and activator of transcription [STAT5], AKT and extracellular signal-regulated kinase [ERK1/2]), in order to promote proliferation and differentiation of erythroid cells and reduce erythroid progenitor's apoptosis. Adapted from Elliot et al. (2014) [203].

3.2. Clinical benefits

The introduction of ESA revolutionized the treatment of anemia in patients with CKD, as they have beneficial effects by correcting anemia and their associated symptoms (fatigue, dizziness, shortness of breath, among others) improving patients' quality of life [37, 204, 205]. ESA also reduced the need for RBC transfusions, thereby reducing transfusion reactions (immunological sensitization), transmission of infectious agents and iron overload [170].

The correction of anemia with ESA is also associated with an improvement on heart failure symptoms and left ventricular hypertrophy (LVH), both in pre-dialysis and dialysis patients [206-208]. LVH is present in many patients with CKD, even in the earlier stages of the disease (75% of patients who start HD have LVH), and may lead to heart failure, cardiac arrhythmia or both, that are considered major causes of cardiac-related deaths in this population [206, 209]. LVH is a physiological adaption that results from long-term increase of myocardial work due to high-pressure or volume overload, which can lead to

major cardiac events. Volume overload can result from anemia, as hypoxia and the decreased blood viscosity contribute to decrease peripheral resistance, and from increased venous return, both of which increase cardiac output [209].

The effects of ESA on the progression of renal function are controversial. Some studies showed that following ESA initiation and correction of anemia renal function declines at a slower rate, delaying the dialysis initiation in pre-dialysis patients [210-212], while other studies reported that ESA do not significantly affect renal function [213, 214]. Therefore, the use of CKD animal models treated with ESA to study the impact of this therapy on renal disease progression would be important.

3.3. Non-hematopoietic actions

ESA are designed to correct anemia, but some evidences showed that ESA (and EPO) present actions beyond hematopoiesis. The non-hematopoietic actions appear to result from the existence of another EPOR receptor (Figure 12), a heterodimeric receptor constituted by an EPOR homodimer complexed with CD131, the common beta receptor (βCR) that is involved in granulocyte macrophage colony-stimulating factor, IL-3 and IL-5 signaling [215]. The two EPOR present different affinities for EPO, as in erythroid cells picomolar concentrations of EPO are sufficient to trigger EPOR homodimer activation, whereas on other cells and tissues high local EPO concentrations are needed to activate EPOR heterodimer [216]. This heterodimer EPOR were detected in several cells and tissues, such as brain (neurons, astrocytes and microglia), kidney, female reproductive system, vascular endothelial cells, cardiomyocytes, lymphocytes and monocytes, among others [217].

Several pleiotropic effects have been attributed to EPO and ESA, such as cytoprotective, anti-apoptotic, anti-inflammatory and angiogenic properties (Figure 12).

EPO and ESA reduce the production of pro-inflammatory cytokines (such as TNF-α, IL-6 and IL-1β) and nitric oxide (NO), via inducible NO synthase (NOS) through the inhibition of NF-κB pathway [218]. Immunomodulator effects of ESA have been described on dendritic cells, therefore presenting effects in innate immunity [219].

EPO seems to be important for the neural development, as it stimulates the differentiation of neural progenitor cells, but it also promotes angiogenesis and reduces inflammation, oxidative stress and neuronal apoptosis in some conditions, including in hypoxia-ischemia, stroke and glutamate neurotoxicity [220]. EPO also increases the number of functionally active endothelial progenitor cells, enhancing angiogenesis, and seems to be dependent on functional endothelial NOS (eNOS) [221].

Several studies showed that EPO/ESA present tissue protective properties. Cardioprotection occurs by the inhibition of cardiomyocyte apoptosis, reduction of

inflammation and oxidative stress, and induction of angiogenesis [222]. In acute lung injury, EPO/ESA present beneficial effects by modulating apoptosis, inflammation, peroxidation, and by inducing angiogenesis [223].

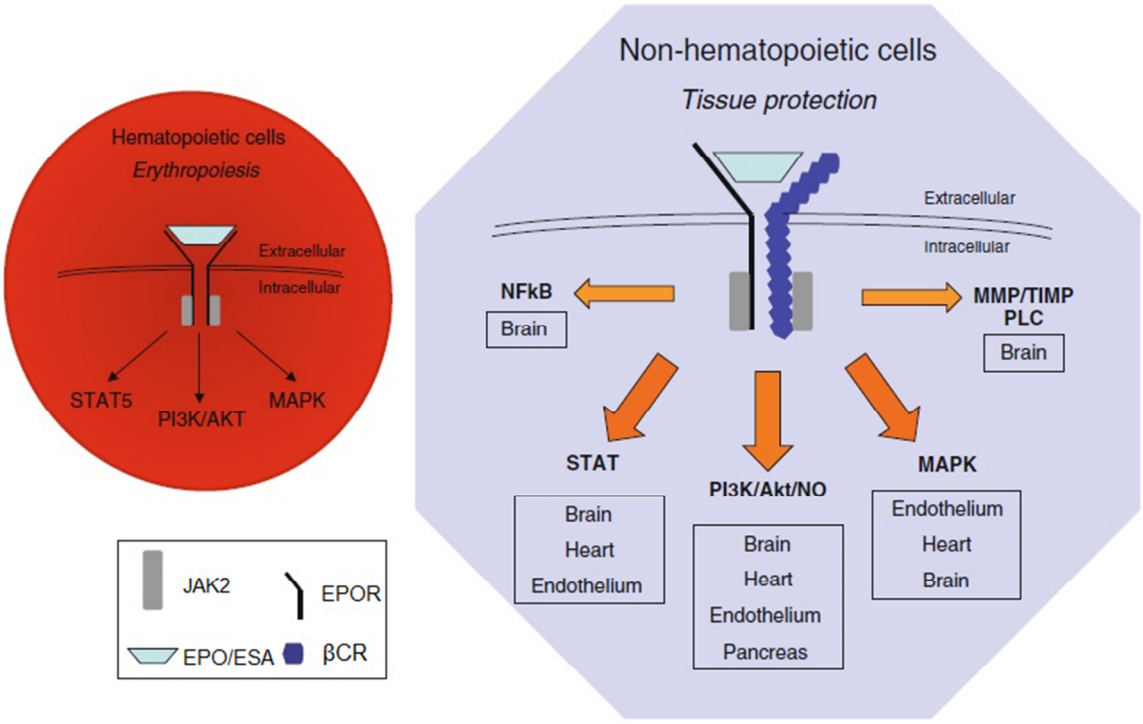


Figure 12 – Signaling pathways activated in hematopoietic (red) and non-hematopoietic (blue) cells.

In hematopoietic cells, erythropoietin (EPO) and erythropoiesis-stimulating agents (ESA) activate the EPO receptor (EPOR) homodimer triggering erythropoiesis, whereas in non-hematopoietic cells, EPO and ESA activate several pathways responsible for tissue protection through the EPOR and common beta receptor (βCR). Adapted from Ogunshola et al. (2013) [217].

The slowing of renal dysfunction observed in some patients may result from the renoprotection afforded by ESA therapy. Several studies on acute kidney injury (AKI) reported that a single dose of rHuEPO reduces kidney dysfunction through an anti-apoptotic mechanism, and increased NO production, but only in intact vessels [224]. ESA therapy also exert renoprotective effects by reducing pro-inflammatory cytokines (IL-1β and TNF-α), acute phase proteins (CRP), pro-fibrotic factors (TGF-β), and oxidative stress [225]. However, it appears that this renoprotection is achieved only with low doses of ESA (non-hematopoietic doses), as high doses cause an increase in hematocrit that is accompanied with changes in hemorheology, activation of thrombocytes and increased platelet adhesion to injured endothelium [224].

Several EPO variants that present the protective effects of EPO in non-hematopoietic tissues, but no hematopoietic activity, have been extensively used to study these pleiotropic effects, namely in animal models, to evaluate putative neuroprotection [226-228].

3.4. Risks

Hypertension is a common side effect associated with ESA therapy [229, 230]. ESA can be responsible for *de novo* or exacerbated hypertension; however, the underlying mechanisms are not fully understood. ESA can induce an increase in blood pressure by hemodynamic alterations, resulting from anemia correction, which is associated with increased RBC mass, hematocrit and, consequently, blood viscosity. These changes increase peripheral vascular resistance, thus, contributing to blood pressure rise [231]. ESA can also act directly on vascular smooth muscle cells (VSMC), promoting its proliferation, which induces hyperplasia of the vascular wall, contributing to increase peripheral vascular resistance and to alterations in renal vessels [232, 233]. ESA appears also to act directly on endothelial cells, stimulating its proliferation and promoting endothelial dysfunction by inducing endothelin-1 expression, a potent vasoconstrictor, and reducing NO synthesis, an important vasodilator [234-237]. Oxidative stress and production of asymmetrical dimethylarginine (ADMA), an inhibitor of eNOS, can also underlie ESA-induced hypertension [238, 239]. ESA also impair the balance between other vasoactive factors, inducing thromboxane A2 (TXA2) and prostaglandin 2α expression (vasoconstrictors), and reducing the production of the vasodilator prostacyclin [240]. The effects on the RAA axis are less clear, as ESA appear to reduce plasma renin levels [196], but an apparent hypersensitivity to angiotensin II has been reported in HD patients [241]. An increase in noradrenaline (another vasoactive substance) concentration and hypersensitivity may also contribute to hypertension during ESA therapy [241].

Thrombotic events have been associated with ESA therapy [242, 243], as it affects thrombopoiesis and platelet function. ESA have the capacity of stimulating thrombopoiesis (increasing platelet count) and platelet reactivity (especially on the newly synthesized ones) promoting a prothrombotic effect [244]. Activated platelets synthesize TXA2, a potent platelet activator, prothrombotic factor and vasoconstrictor, and high levels were found after ESA treatment, as well as increased E-selectin levels [245]. Some other hemostatic disturbances have been described, as an increased expression of P selectin, von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1), which may increase the risk of thrombosis and thromboembolism, as occlusion of the vascular access [240].

In the treatment of anemia secondary to chemotherapy in adult cancer patients with non-myeloid malignancies (Table 8), some evidences indicate that ESA can accelerate tumor growth, by promoting angiogenesis; the anti-apoptotic pleiotropic effect of ESA can also contribute to tumor progression [246].

A rare but serious complication associated with ESA treatment is pure red blood cell aplasia (PRCA), an immunogenic side-effect that results from the production of anti-rHuEPO antibodies, directed against the protein moiety of the molecule [247-249]. The method used to produce ESA may not eliminate impurities or aggregated protein that may trigger the immune response in patients [249].

Despite all the benefits associated with ESA therapy, some adverse effects have been associated with this therapy. As described above, some effects of EPO and ESA are beyond the correction of anemia, and seem to be achieved by the use of increasing ESA doses, needed to trigger the activation of EPOR heterodimer. The current KDIGO guidelines [48] are in line with this, by recommending a more conservative increase in ESA dose used in CKD patients.

3.5. Hyporesponsiveness

The majority of CKD patients respond adequately to ESA, but 5-10% of them do not respond properly to this therapy [50]. According to the KDIGO guidelines [48], CKD patients can present initial or acquired ESA hyporesponsiveness: primary hyporesponsiveness is present when a patient has no increase in Hb concentration after one month of treatment with adequate weight-based ESA dose, whereas acquired ESA resistance exists, if after treatment with stable ESA dose used to maintain Hb concentration, a patient requires 2 consecutive increases (up to 50% beyond the stable dose) in ESA dose.

Hyporesponsiveness (also widely referred as resistance) to ESA therapy is associated with a poor outcome, progression of renal disease, sudden death, infectious complications and all-cause mortality, mainly in dialysis patients [250-254]. Several causes are associated with poor response to ESA therapy, including iron deficiency, inflammation, malnutrition, hyperparathyroidism, among others [255-258]. ESA hyporesponsiveness is typically a transient condition, and correction of its treatable causes (Table 10) is usually sufficient to overcome this state, but careful monitoring of these patients should be continued [48].

Higher doses of ESA are needed to correct anemia in African-American patients compared to Caucasians subjects, due to the highest prevalence of sickle cell disease and of other hemoglobinopathies in that population [259]. Age and gender can also influence response to ESA therapy. Children and older age patients frequently need higher ESA dose

to achieve target Hb values [255, 260]. Female patients often need to use higher ESA dose, due to menstrual blood loss. In males, the androgenic stimulation of erythropoiesis through the production of hematopoietic growth factors improves iron bioavailability, and, thus, erythropoiesis [260]. In this sense, low testosterone levels in males with CKD may favor anemia and ESA resistance [261].

Table 10 – Potentially correctable and non-correctable factors involved in erythropoiesis-stimulating agents response

Easily correctable	Potentially correctable	Impossible to correct
Absolute iron deficiency	Infection/Inflammation	Hemoglobinopathies
Vitamin B12 deficiency	Underdialysis	Bone marrow disorders
Folate deficiency	Hemolysis	
Hypothyroidism	Bleeding	
ACE inhibitor/ARB	Hyperparathyroidism	
Non-adherence	Pure red cell aplasia	
	Malignancy	
	Malnutrition	

Abbreviations: ACE - Angiotensin-converting-enzyme; ARB – Angiotensin-receptor blocker. Adapted from KDIGO group (2012) [48].

3.5.1. Iron deficiency/overload

Iron deficiency is present in about 60% of patients with creatinine clearance <60 mL/min [262]. In CKD patients this condition may be due to increased iron demand to the bone marrow induced by ESA to sustain erythropoiesis; in HD patients the chronic blood loss is another factor contributing to iron deficiency. Supplementation with iron (oral or IV) can overcome absolute iron deficiency. Functional iron deficiency is also a frequent entity in CKD patients, mainly in those on dialysis, and in ESA resistance, due to iron restriction to erythropoiesis, by impaired iron release from iron stores [263] that, usually, can be corrected by IV iron supplementation [264, 265]. Hepcidin levels, particularly increased in HD patients [31, 266], are associated with functional iron deficiency [263], and could be clinically useful to assess iron status [267]. As already referred, hepcidin can be increased by inflammation [93], a common feature in CKD patients [31, 90], but it can be also increased due to reduced urinary excretion. Hepcidin is also found in urine

indicating that the kidney may play a role in the excretion of this peptide [91] and, therefore, the blood hepcidin levels might be influenced by residual kidney function [268].

In the recent years, the proportion of patients with serum ferritin values ≥800 ng/mL has increased [269] and several studies reported tissue iron accumulation (liver and spleen) in CKD patients [270-273], which may contribute to increase hepcidin synthesis and further aggravate ESA hyporesponsiveness. The effects of iron overload will be discussed in another chapter (4.1. Iron therapy).

3.5.2. Inflammation

CKD is considered a pro-inflammatory state, mainly due to increased uremic toxins that induce the production of inflammatory cytokines; however, other causes can also contribute to inflammation, such as active infections, vascular access for HD procedure and surgery-evoked inflammation (vascular surgery included). In this sense, the use of dialysis catheter is associated with higher inflammatory features, compared with the use of arteriovenous fistula [31, 260].

Elevated pro-inflammatory cytokines are associated with enhanced oxidative stress, responsible for alterations in RBC membrane and activation of macrophages, increasing erythrocyte damage and reducing its lifespan [274]. Uremic toxins and pro-inflammatory cytokines also inhibit bone marrow erythropoiesis, as already referred above, and may favor ESA hyporesponsiveness. A weak response to ESA also appears to be associated with enhanced T cell capacity to express IFN-γ, TNF-α, IL-10, and IL-13 [90, 275]. Inflammation also induces hepcidin synthesis limiting iron availability for erythropoiesis.

In patients with iron replete stores, inflammation is the best predictor of ESA response, and IL-6 and CRP appear to be the best markers [250, 276-278]. Neutrophil count is increased in poor responders to ESA therapy, and positive correlations were found between CRP and elastase, and between elastase and rHuEPO doses, suggesting that elastase, a neutrophil protease released by degranulation, could be a good marker of resistance to ESA therapy in CKD patients under HD [279].

3.5.3. Malnutrition

Low body mass index, low levels of total cholesterol and triglycerides are related to poor outcomes in dialysis patients, increasing the risk of mortality [31, 280]. This phenomenon is called “reverse epidemiology” as the reduction in these factors is associated with a lower CVD risk in the general population. CKD patients present a malnutrition-inflammatory complex; thus, when they present a decreased nutritional reserve, their capacity to overcome inflammation seems to be reduced. A reduced protein-

calorie intake, chronic acidosis and failure of the vascular access, all contributing to requirement of higher ESA dose to correct anemia [281], may also underlie this reverse epidemiology.

3.5.4. *Secondary hyperparathyroidism*

The parathyroid hormone (PTH) is considered by EUTox Work Group as an uremic toxin with some biological effects [153]. Secondary hyperparathyroidism is a condition that results from the dysregulation of calcium and phosphorus homeostasis in the kidney. It seems that PTH could be a marker of hyporesponse to ESA in dialysis patients [282, 283], considering that PTH causes bone marrow fibrosis, presents an inhibitory effect on BFU-E, interferes with endogenous EPO and increases osmotic fragility of erythrocytes, thus, interfering with RBC production and removal [284-286].

3.5.5. Aluminium toxicity

Despite the recent progresses in the dialysis procedures, some patients still present high levels of aluminium [260]. Usually, high levels of aluminium cause a microcytic, hypochromic or normochromic anemia that is hyporesponsive to ESA therapy, as it interferes with the enzymes necessary for heme synthesis. The sources for the increase in plasma aluminium levels seem to be the water used for dialysis, IV medications and infections [287, 288].

3.5.6. Inadequate dialysis

The intensity or adequacy of dialysis is a factor that can modulate the response to ESA therapy. Inadequate dialysis is associated with the need for higher ESA doses. Some studies showed that convective treatments present benefits in ESA response, as compared with other treatments [289]. High flux HD and online hemodiafiltration improve the response to ESA, as compared to low flux HD, probably due to a better removal of middle and large molecules that impair erythropoiesis [289]; however, some studies failed to reach to the same conclusions [290, 291].

3.5.7. Vitamins deficiency

The deficiency of folate or vitamin B12 is not very common in dialysis patients, but as these nutrients are water soluble and can be easily loss during dialysis, they can become a cause of ESA hyporesponse, especially in patients with malnutrition. The supplementation of these nutrients seems to overcome ESA hyporesponsiveness [260].

Vitamin D deficiency is now a recognized cause for ESA resistance in some patients due to reduced kidney capacity to synthesize the active form of vitamin D. It is known that

vitamin D is important for bone mineral metabolism, but the link between vitamin D and ESA response seems to result from a direct stimulatory action on erythroid progenitors or the modulation of innate and adaptive immunity, controlling the expression of inflammatory cytokines and, therefore, reducing the effects of inflammation [292].

3.5.8. Drugs

ACE inhibitors and angiotensin-receptor blockers, used to treat hypertension, can be associated with ESA hyporesponsiveness due to their effects on angiotensin II. They can act through several mechanisms, not well understood, including inhibition of angiotensin-induced EPO release and increment in plasma levels of N-acetyl-serylaspartyl-lysil-proline that impairs the recruitment of pluripotent hemopoietic stem cells [260].

4. CONTROVERSIES IN THE TREATMENT OF ANEMIA IN CHRONIC KIDNEY DISEASE

4.1. Iron therapy

One of the concerns in IV iron therapy is that the used iron dose overwhelms the binding capacity of Tf, leading to an increase in non-Tf bound iron (NTBI). Moreover, as Tf levels are usually reduced in CKD patients, this effect might be enhanced [31, 89]. The presence of NTBI can lead to the development of oxidative stress, increasing the pro-oxidant state of these patients. In the case of co-existence of inflammation and CVD, the oxidative stress may be even more enhanced. It has been also suggested that NTBI might be a source for iron deposition in organs [293].

The Dialysis Outcomes and Practice Patterns Study (DOPPS) [269] showed that the number of HD patients with IV iron supplementation increased in most countries; and even more important, the doses prescribed to these patients also increased in the past 10-15 years. Overall, the mean serum ferritin has increased overtime, and the proportion of patients with serum ferritin values ≥800 ng/mL has also increased [269].

The recent KDIGO Controversies Conference on Iron Management in CKD (San Francisco, 2014) recognized the entity of iron overload in HD patients. Actually, there are several studies (Table 11) reporting tissue iron accumulation in HD patients treated with ESA and IV iron. Canavese et al. [270] found that 70% of HD patients present mild-to-severe hepatic iron overload, although the same percentage of patients presented serum ferritin values below 500 ng/mL. Ferrari et al. [271] reported that 60% of HD patients present liver iron concentration >60 μmol/g (above the normal upper limit of 30 μmol/g) and 13% had liver iron concentrations >130 μmol/g, which is usually found in patients with hemochromatosis; they also found that liver iron accumulation was correlated with cumulative iron dose, but no correlation was observed with ferritin or TSAT. Recently, two other studies found similar results [272, 273]. Ghoti et al. [272] reported that 90% of HD patients present mild to severe hepatic iron deposition and also iron deposition in the spleen. In the other study [273], 84% of the HD patients had hepatic iron overload, among which 30% presented severe iron overload; moreover, liver iron content correlated with infused iron.

In spite of the widespread use of IV iron supplementation in HD patients, the safest dosing strategy is still poorly clarified, as well as its relation with serum ferritin levels, iron overload and mortality risk. Actually, only few studies addressed these questions, and presented controversial results (Table 11). Feldman et al. [294] found that HD patients receiving more than 1000 mg of IV iron in a period of 6 months presented a higher risk of death and hospitalization, compared to patients with no iron supplementation. In a

follow-up study of 2 years, the same group evaluated the effect of each 6 months of iron exposure and did not find any association between the iron administrated and mortality, but found an association between iron dose and mortality at 12 to 18 months of treatment, for iron doses higher than 1800 mg [295]. In the first study of this group [294], only 17% of the HD patients received iron dosing above 1000 mg, whereas in the second study [295] this number rose to 48%. These authors also found an association between mortality and ferritin levels >800 ng/mL in the 6 months prior to death, but this finding could result from confounding factors, such as inflammation and malnutrition [295].

Another observational study evaluating HD patients for a period of 2 years showed that serum ferritin levels in the range of 200-1200 ng/mL, TSAT between 30 and 50% and IV iron dose <400 mg by month were associated with improved survival, suggesting that the association of high ferritin levels and mortality could also result from confounding factors [296].

A recent study [297] evaluated the effect of a bolus of IV iron (consecutive doses ≥100 mg exceeding 600 mg during one month) *versus* maintenance of IV iron therapy (all other iron doses during the month), and also the effect of high (>200 mg over 1 month) *versus* low dose of IV iron (≤200 mg over 1 month); after 1 month of iron exposure, the patients were studied for a follow-up period of 3 months; no significant associations of bolus *versus* maintenance dose or of high *versus* low dose IV iron with increased short term cardiovascular morbidity and mortality were found. Zitt et al. [298] found that increasing ferritin concentrations (>800 ng/mL) in patients with normal CRP concentrations were associated with decreasing mortality, whereas in patients with high CRP values (>5 mg/dL) the increasing ferritin concentration was linked with increased mortality, suggesting that serum ferritin levels above 800 ng/mL are associated with increased mortality in the case of concomitant inflammation. A 2-year follow-up study, by our group [31], found that HD patients present several changes in iron metabolism (increased TSAT, ferritin and hepcidin and a decrease in iron and Tf) and in inflammatory markers (increased IL-6 and CRP). Patients who died during this follow-up period showed significantly lower values for Tf and TSAT and increased levels of IL-6 and CRP. In the adjusted survival regression model, CRP was found to be a significant predictor of mortality.

The recently published observational DOPPS study [299] showed that HD patients receiving, along 4 months, 300 mg/month or even a higher dose of IV iron supplementation, compared with patients supplemented with 100-199 mg/month, presented increased hospitalization risk, all-cause mortality and cardiovascular mortality, regardless of serum ferritin or CRP concentrations.

Table 11 – Studies evaluating iron overload and mortality risk associated with intravenous iron therapy in dialysis patients

	Year	Patients	Iron dose/follow-up time	Outcome	Ref.
Iron overload	2012	HD patients (n=119)	100 mg of iron sucrose (2-3x/w - induction phase; 1x/w every 4 w - maintenance phase) 60 mo follow-up	• 84 % of patients with hepatic iron deposition, 30% of them with severe iron overload • Iron liver content correlates with infused iron	[273]
	2012	HD patients (n=21)	100 mg (ferric saccharate) 1-3x/w 12 mo follow-up	• 90% of patients with mild-to-severe hepatic iron deposition • 95% of patients with splenic iron deposition • 14% patients with pancreatic iron deposition	[272]
	2011	CKD patients (n=25)	50 to 200 mg/mo for 12 mo	• Liver iron concentration: 60% of patients > 60 umol/g; 13% of patients > 130 umol/g (reference value 30 umol/g)	[271]
	2004	HD patients (n=40)	31.25 mg ferric gluconate complex 10 patients: maintenance iron at least 6 mo 30 patients: without iron therapy at least 2 mo (after ferritin >500 ug/L)	• 70% of patients with mild-to-severe hepatic iron overload • 70% of patients with ferritin < 500 ng/mL	[270]
Mortality	2015	HD patients (n=32 435)	4 month-follow up of IV iron dose: < 300 mg/mo *versus* ≥ 300 mg/mo	• ↑ mortality among patients with higher doses of IV iron (≥ 300 mg/mo over 4 months), regardless serum ferritin or CRP values	[299]
	2014	dialysis patients (n=235)	7.6 years-follow up with continuous maintenance iron therapy once/w in varying doses: 12.5 mg (minimum dose) to 62.5 mg (maximum dose)	• ↑ mortality with ferritin levels > 800 ng/mL in case of concomitant inflammation (CRP > 0.5 mg/dL)	[298]
	2013	HD patients (n=117 050)	3 mo-follow up after 1 mo exposure to: high dose (> 200 mg) *versus* low dose (1-200 mg) bolus (consecutive doses ≥ 100 mg exceeding 600 mg during one month) *versus* maintenance dose	• no significant associations of bolus *versus* maintenance dose or high dose *versus* low dose IV iron with increased short-term cardiovascular morbidity and mortality	[297]
	2005	HD patients (n=58 058)	Iron gluconate effect in a 2 year-follow up, with different iron dose categories: 0 mg/mo; 1 to 199.9 mg/mo; 200 to 399.9 mg/mo; > 400 mg/mo	• ferritin 200-1200 ng/mL, TSAT 30-50% and IV iron dose < 400 mg/mo associated with improved survival	[296]
	2004	HD patients (n= 27 280)	Effect of iron administration in a 2 year follow-up, with iron doses categories at each 6 mo: >0 to 700 mg; > 700 to 100 mg; > 1000 to 1800 mg; > 1800mg	• no association between iron administrated and mortality • association between iron dose and mortality at 12 to 18 months of treatment, for doses > 1800 mg • association between mortality and ferritin > 800 ng/mL in the 6 months prior to death	[295]
	2002	HD patients (n= 10 169)	number of 100-mg vials of iron during 6 mo	• ↑ risk of death and hospitalization with IV iron > 1000 mg	[294]

Abbreviations: CRP — C reactive protein; HD — hemodialysis; IV — intravenous; mo — month; w — week. Adapted from Ribeiro et al. (2015) [84].

Considering the controversial data in literature, there is a clear need to develop clinical trials with longer follow-up periods of HD patients to evaluate the effect of long-term cumulative IV iron doses and the impact of serum ferritin levels on all-cause and cardiovascular mortality. In accordance, a clinical trial, "The Proactive IV irOn Therapy for HaemodiALysis patients (PIVOTAL)", has recently started (2013), and the aim is to compare the effect of IV iron high-dose *versus* low-dose regimen on all-cause mortality, and to evaluate the incidence of non-fatal cardiovascular endpoints, in HD patients along 2-3 year follow-up [300]. Another aim of this study is to compare the effect of the two regimens on ESA dose requirements, RBC transfusions, complications of HD treatment, and quality of life of the patients.

Iron toxicity is also a concern surrounding iron therapy. Data in literature strongly suggest the risk of tissue iron overload in HD patients, although the underlying pathophysiological mechanisms are less clear, especially in the case of IV administration of iron. As referred, the infusion of iron may overwhelm the capacity of the iron binding proteins, allowing iron to become free in circulation and/or to increase iron stores. It is known that free iron can react with hydrogen peroxide leading to the production of hydroxyl radicals that are able to trigger oxidative modifications in lipids, proteins and DNA. Indeed, the literature supports that after IV iron injection in HD patients a transient increase in oxidative stress occurs, as shown by the increase in plasma lipid peroxidation [301] and oxidative modification of proteins [302]. Actually, different markers of oxidative stress are significantly increased in CKD patients [303, 304] and are involved in the progression of renal disease [305].

Several diseases have been associated with oxidative stress, such as CVD [306, 307], which are the major cause of death in HD patients. It has been hypothesized that the oxidative stress induced by IV iron infusion could favor atherosclerosis and endothelial cell damage [308, 309]. NTBI might be important for extrahepatic iron deposition and toxicity, namely, in the kidney. Progressive tubulointerstitial damage and renal fibrosis are common pathways in the development of CKD and iron deposition could favor these lesions. Indeed, iron accumulation is observed in the proximal tubule in human CKD [310], as well as in rats with nephropathy [311-313], and seems to be associated with the progression of CKD [311]. In a study by our group, using a rat model of nephrectomy, we found iron deposition in tubules, along with extensive tubulointerstitial lesions, after 9 weeks of nephrectomy [313].

An association of iron therapy with infection has been inconsistently reported in literature. One study observed a significantly higher rate of bacterial infection with higher IV iron saccharate dose, but not with higher frequency of dosing administration [314]. A one year follow-up study of HD patients examined the relationship between iron stores, IV

iron dosing and bacteremia risk, and found that patients with higher iron stores had a significantly higher risk of bacteremia; however, they did not find an IV iron dose-response relationship [315]. Brewster et al. [316] reported that IV iron did not significantly increase the rate of microorganism growth within catheters or the development of blood infections with iron supplementation. After the recent changes in the pattern of IV iron treatment [48], Bansal et al. [317] in a 2 year follow-up study of HD patients treated with IV iron found no increase in the incidence of infectious complications. In a retrospective cohort study of HD patients, those receiving bolus *versus* maintenance iron therapy were at increased risk of infection-related hospitalization [297]. Recently, Kuragano et al. [318] found that patients with a higher ferritin level had a higher risk of infectious disease than those with lower ferritin level. They also found that the risk of infection and hospitalization were significantly higher among patients who were treated with high weekly doses of IV iron, compared with no iron. An observational study that included 14 078 patients reported the possibility of infection-related mortality with higher iron doses [319].

The type of IV iron formulation might also have an impact in the rate of infection, as suggested by Sirken et al.[320] that found a higher bacteremia rate with iron sucrose than with ferric gluconate; however, a relationship between IV iron dose and bacteremia was not supported. Randomized clinical trials are needed to assess the effect of cumulative IV iron doses and the risk of infection-related mortality.

4.2. High hemoglobin target or high erythropoiesis-stimulating agents dose

Despite all the benefits of ESA therapy some concerns have been raised, due to studies reporting a high incidence of cardiovascular events and mortality in CKD patients treated with ESA [243, 321], though no difference on mortality risk was observed for the different ESA available [322-324].

Since the introduction of ESA therapy a demand exists to define the better Hb target associated with lower cardiovascular risk. Indeed, recent studies reported increased cardiovascular risk and death in patients treated with high ESA doses to achieve higher Hb levels. This led to the controversy surrounding increased risk: if higher doses of ESA or higher levels of Hb. Only four studies assessed properly the effect of higher Hb levels on the increased risk of cardiovascular events and/or death.

The Normal Hematocrit Trial [325] included patients under HD with congestive heart failure or ischemic heart disease. HD patients were randomized to receive epoetin alpha aiming to achieve and maintain a target Ht of 42% (high-Ht group) or 30% (normal-

Ht group). Primary end points were the length of time to death or to first nonfatal myocardial infarction. The study was interrupted due to the increased number of deaths observed in the high-Ht group and that were nearing the boundary of statistical significance. An increased incidence rate of vascular access thrombosis was also reported in the high-Ht group. The study failed to reach statistical difference between the two groups, however, it was concluded that a target Ht of 42% is not recommended in HD patients.

In 2006, the Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta (CREATE) [326] included pre-dialysis patients in stage 3 or 4 with mild-to-moderate anemia, randomly assigned to normalization of Hb values (13.0-15.0 g/dL) or to a partial correction of anemia (10.5-11.5 g/dL), in order to investigate the effect of Hb correction on complications from cardiovascular causes. The primary endpoint was the time to first cardiovascular event; secondary objectives included the investigation of the effects of these treatments on the left ventricular mass index, the progression of CKD and the quality of life. A significant difference in the risk for a first cardiovascular event between the two groups was not found. However, this study reported a higher incidence of hypertension and headaches, and a higher risk for starting dialysis in the group aiming to normalize Hb values. On the other hand, it was reported significant benefits on the quality of life for the patients with higher Hb targets. It was concluded that in pre-dialysis patients with mild-to-moderate anemia, the normalization of Hb levels to 13.0-15.0 g/dL does not reduce cardiovascular events.

The Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) study [327], published in the same year of the CREATE study, included ND-CKD patients and the effect of raising Hb concentration with epoetin alpha to a target Hb value of 13.5 g/dL (high-Hb group) or 11.3 g/dL (low-Hb group) was compared. The primary end point was the time of death, myocardial infarction, hospitalization due to congestive heart failure (excluding renal replacement therapy), or stroke. An increased risk for the primary end point was found in the high-Hb group, as compared with the low-Hb group. Death and hospitalization due to congestive heart failure accounted for 74.8% of the events. An increased rate of thrombotic events was also reported in the group aiming high-Hb levels. Patients in the high-Hb group had a higher (but not significant) rate of both progression and hospitalization to renal replacement therapy. The study found no apparent additional benefit in quality of life. In conclusion, it was recommended the use of a target Hb level of 11.0-12.0 g/dL rather than a level of 11.0-13.0 g/dL to correct anemia in these patients, because of the increased risk, increased costs, and no quality-of-life benefit.

Considering the results of these two studies (CHOIR and CREATE), in 2007, the FDA launched a safety advisory, recommending that patients should not exceed the Hb

level of 12 g/dL [328]. At the same time, the NKF KDOQI made an update on its guidelines, recommending that the selected Hb target should generally be in the range of 11.0-12.0 g/dL, but should not be higher than 13.0 g/dL [329]. In 2010, the ERBP Work Group published the recommendation that "Hb values of 11-12 g/dL should be generally sought in the CKD population without intentionally exceeding 13 g/dL" [330].

In 2011, the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) was published [42]. In this clinical trial patients with type 2 diabetes mellitus, CKD and anemia were enrolled and were randomized to receive darbepoetin-alfa (in order to achieve a target Hb of 13.0 g/dL) or placebo (in this group was prescribed blinded "rescue" darbepoetin for Hb level <9.0 g/dL). The primary end point was time to death or hospitalization due to myocardial ischemia. A significantly higher rate of strokes in patients treated with darbepoetin was observed. A higher rate of both thromboembolism and cancer-related deaths among patients with a history of cancer in the treatment group was also reported.

After the TREAT study was published, FDA introduced warnings in the ESA label giving the recommendations "for more conservative dosing of ESA in patients with CKD to improve the safe use of these drugs" [331]. This led to the revision of the anemia treatment guidelines and, in 2012, the KDIGO guidelines reaffirmed the use of more conservative doses [48], that was further translated to the ERBP guidelines [332].

Several potential harmful mechanisms for higher Hb targets have been proposed, as increased blood viscosity and hemoconcentration, the increased blood pressure, the toxic effect of iron and non-physiological doses of ESA, which contribute to ESA toxicity. The rise in Ht results in a higher viscosity and, consequently, higher risk of thromboembolism. It also favors platelet activation by increasing the interaction between the endothelial cells and platelets in blood vessels. Hemoconcentration is a phenomenon observed in HD patients after a dialysis session that results from the removal of large amounts of fluids [333].

In conjugation with the optimal Hb target and ESA dose, Hb variability must be taken into account, as it was noted that during the treatment of HD patients with ESA the level of Hb has a large fluctuation; that is, the Hb level tends to rise or fall in a cyclic pattern, which is different for each patient [334]. Some studies showed an association between Hb variability and increased risk of death, mainly due to cardiovascular events, infection, and hospitalization [318, 335-337]. Hb variability represents an important physiological stress, as the ESA treatment involves short, intermittent burst of plasma EPO availability that do not coincide, either temporally or in magnitude, with its physiological action. Under physiological conditions EPO levels are maintained in a narrow range (through several mechanisms), in order to support a constant oxygen supply

to the organs. The impact of Hb variability on the organism is not fully understood, but the myocardium may be one of the most affected organs, as it has to compensate with an increased output and cardiomyocytes proliferation during the periods of reduced oxygen availability, that occur when Hb reaches lower levels, before the new ESA administration [334, 335].

Nevertheless, the debate about the cause of this increased risk with higher doses of ESA or higher levels of Hb remains controversial. Naturally occurring Hb levels >12 g/dl does not associate with increased mortality among HD patients, suggesting that high Hb are not always harmful in CKD patients [338]. In a meta-regression analysis it was reported that in CKD patients, higher ESA dose might be associated with all-cause mortality and cardiovascular complications independent of Hb level [339]. A secondary analysis of the CHOIR study showed that high-dose of ESA was associated with a significant increase hazard of a primary end point [340]; however, it is also true that the patients that require higher ESA dose were those that present the inability to achieve the target Hb, which could be related to CKD and anemia complications, such as inflammation and malnutrition [341]. Indeed, a secondary analysis to the TREAT study showed that in diabetic CKD patients, both lower baseline GFR and higher CRP levels are associated with higher cardiovascular and non-cardiovascular mortality rates, particularly from sudden death and infection [342]. We cannot also forget that higher ESA doses are associated with some risks, as above mentioned, in agreement with the report of Koulouridis et al. (2013) [339], where mean ESA dose was associated with increased rate of hypertension, stroke, and thrombotic events, including dialysis vascular access-related thrombotic events. To address if high ESA doses are associated with increased mortality risk an ongoing trial has been designed. The Clinical Evaluation of the DOSe of Erythropoietins (C.E. DOSE) trial has been designed to identify the potential benefits and harms of different fixed doses of ESA in HD patients that were randomized 1:1 to 4000 IU/week *versus* 18000 IU/week of IV epoietin alfa or beta, or of any other ESA in equivalent doses. The primary outcome was death, non-fatal stroke, non-fatal myocardial infarction and hospitalization for cardiovascular causes, but no results were yet published [343].

AIMS

Anemia is a common complication in CKD patients, mainly due to the reduced capacity of the failing kidneys to synthesize EPO, an essential hormone for erythropoiesis. The correction of anemia is achieved by the use of ESA; however, some patients do not respond properly to ESA therapy, developing hyporesponsiveness, with several potential underlying causes, such as inflammation and iron disturbances. ESA hyporesponsiveness is usually associated with poorer outcome, progression of renal disease and increased mortality. To overcome this resistance higher doses of ESA are usually needed; however, recent studies reported an increased risk for cardiovascular events and mortality rate in patients treated with higher ESA doses, whose specific causes and mechanisms remain to be elucidated.

In this sense, the main aims of this research project were:

1. To prepare a critical up-to-date revision regarding the benefits and risks of ESA and iron therapy in CKD patients.

   Book chapter - *rhEPO for the treatment of erythropoietin resistant anemia in hemodialysis patients - Risks and Benefits* - Appendix I

   Review article - *Iron therapy in chronic kidney disease: recent changes, benefits and risks* - Appendix II

2. To use the remnant kidney rat model of chronic renal failure (CRF) induced by 5/6 surgical nephrectomy, to characterize anemia development, renal function and damage, blood pressure, iron metabolism and the crosstalk between the induced disturbances

   Paper I - *Renal risk-benefit determinants of recombinant human erythropoietin therapy in the remnant kidney rat model – hypertension, anemia, inflammation and drug dose*

3. To evaluate the impact of different rHuEPO doses on anemia correction, renal damage/disease progression, inflammation, iron disturbances and blood pressure. In this sense, CRF animals were treated during 3 weeks with standard rHuEPO doses, usually used to correct anemia in CKD patients (100 and 200 IU/Kg body weigh [BW]/week), and with higher doses (400 and 600 IU/Kg BW/week), used in the treatment of hyporesponsive patients.

Paper I and **Paper II** - *Liver iron is a major regulator of hepcidin gene expression via BMP/SMAD pathway in a rat model of chronic renal failure under treatment with high rHuEPO doses*

4. To dissect the pathological and molecular mechanisms underlying rHuEPO hyporesponsiveness in this animal model, focusing on anemia, iron metabolism, renal damage, hypoxia, inflammation and fibrosis.

   Paper III - *Pathological and molecular mechanisms underlying resistance to recombinant human erythropoietin therapy in the remnant kidney rat model of chronic kidney disease associated anemia*

5. To clarify the effect of rHuEPO therapy, *per se* (in normal rats, without CRF), on erythropoiesis, renal function and damage, blood pressure and iron metabolism.

   Paper IV - *Impaired renal endothelial nitric oxide synthase and reticulocyte production as modulators of hypertension induced by recombinant human erythropoietin in the rat*

   Paper V - *Recombinant human erythropoietin-induced erythropoiesis regulates hepcidin expression over iron status in the rat*

RESULTS

Paper I

Renal risk-benefit determinants of recombinant human erythropoietin therapy in the remnant kidney rat model – hypertension, anemia, inflammation and drug dose

Sandra Ribeiro, Patrícia Garrido, João Fernandes, Helena Vala, Petronila Rocha-Pereira, Elísio Costa, Luís Belo, Flávio Reis and Alice Santos-Silva

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Renal risk-benefit determinants of recombinant human erythropoietin therapy in the remnant kidney rat model – hypertension, anaemia, inflammation and drug dose

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SUMMARY

Clinical studies showed that high doses of recombinant human erythropoietin (rHuEPO) used to correct anaemia in chronic kidney disease (CKD) hyporesponsive patients may lead to deleterious effects. The aim of this study was to analyze the effects of rHuEPO in doses usually used to correct CKD-anaemia (100, 200 IU/kg body weight [BW] per week) and in higher doses used in the treatment of hyporesponsive patients (400, 600 IU/kg BW per week), focusing on renal damage, hypoxia, inflammation and fibrosis. Male Wistar rats with chronic renal failure (CRF) induced by 5/6 nephrectomy were treated with rHuEPO or with vehicle, over a 3-week period. Haematological, biochemical and renal function analyses were performed. Kidney and liver mRNA levels were evaluated by quantitative real-time polymerase chain reaction (qPCR) and protein expression by Western blot and immunohistochemistry. Kidney histopathological evaluations were also performed. The CRF group developed anaemia, hypertension and a high score of renal histopathologic lesions. Correction of anaemia was achieved with all rHuEPO doses, with improvement in hypertension, renal function and renal lesions. In addition, the higher rHuEPO doses also improved inflammation. Blood pressure was reduced in all rHuEPO-treated groups, compared to the CRF group, but increased in a dose-dependent manner. The current study showed that rHuEPO treatment corrected anaemia and improved urinary albumin excretion, particularly at lower doses. In addition, it is suggested that a short-term treatment with high doses, used to overcome an episode of hyporesponsiveness to rHuEPO therapy, can present benefits by reducing inflammation, without worsening of renal lesions; however, the pro-hypertensive effect should be considered, and carefully managed to avoid a negative cardiorenal impact.

Key words: anaemia, CKD, high rHuEPO doses, hypertension, hyporesponsiveness, inflammation.

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INTRODUCTION

Chronic kidney disease (CKD) is a worldwide health problem with increasing incidence and prevalence rates.[1] Loss of renal function is associated with inflammation,[2] compromising oxygen (O_2) availability and worsening hypoxia-induced anaemia, a common complication in CKD patients. Renal hypoxia promotes fibrosis by stimulating renal cells to produce extracellular matrix, thus contributing to renal disease progression.

Anaemia occurrence and severity increases with the progression of renal disease.[3] Although it is accepted that anaemia of CKD patients is mainly due to the reduced erythropoietin (EPO) production by the failing kidneys,[2] the ability of the kidney to synthesize EPO, as well as the liver contribution to EPO production, remains to be clarified.

Erythropoiesis-stimulating agents (ESA) are the cornerstone therapy to anaemia correction, improving CKD patients' quality of life.[4] While some authors claim that ESA treatment delays renal disease progression,[5, 6] others reported that ESA therapy does not significantly slow renal function decline.[7, 8] Preclinical data from animal models of CKD treated with ESA also showed controversial results; in fact, while some studies reported the ability to correct anaemia without a positive impact on renal disease progression,[9, 10] others described ample beneficial effects.[11, 12] This controversy is most probably due to non-standardized protocols, with different ESA doses and/or duration of treatments.

Tissue-protective effects attributed to EPO are mediated by a heterodimer receptor, consisting of EPO receptor (EPOR) and CD131 (beta common receptor), that needs higher doses to be activated.[13, 14] It is well known that there is a marked variability in CKD patients' response to ESA treatment; with 5–10% becoming resistant to therapy.[15] To overcome this hyporesponse, higher doses of ESA are needed for anaemia correction; however, these patients present higher rates of cardiovascular events and mortality.[16–18] These adverse effects have been associated with the increase in haemoglobin (Hb) concentrations and/or ESA doses used to achieve target Hb levels. Recently, the *post hoc* analysis of the TREAT study reported that CKD patients with poor initial haematopoietic response to ESA therapy, therefore needing, higher ESA doses, presented increased risk for adverse cardiovascular events and death, thus suggesting that the adverse outcome might be mainly due to the higher ESA doses used to achieve the target Hb values.[19]

The present work aimed to study the effects of recombinant human erythropoietin (rHuEPO) treatment at doses usually used to correct anaemia (100 and 200 IU/kg body weight [BW] per week) in rHuEPO-sensitive CKD patients and at higher rHuEPO doses (400 and 600 IU/kg BW per week), used in hyporesponsive CKD patients. In particular, this experimental study intended to access, using the remnant kidney rat model of chronic renal failure (CRF) induced by 5/6 nephrectomy, the crosstalk between CKD-associated anaemia and renal damage; in addition, to evaluate the effects of a short-term rHuEPO treatment on kidney disease progression, focusing on renal damage, hypoxia, inflammation and fibrosis.

RESULTS

Haematological and biochemical data

Haematological data at baseline (0w), after one (1w) and three weeks (3w) after starting rHuEPO treatment (end of protocol) are presented in Table 1.

The CRF group presented a significant reduction in red blood cell (RBC) count, Hb concentration and haematocrit (Ht) values, one week after nephrectomy, when compared to the Sham group. These alterations remained until the end of protocol

Table 1 Haematological data throughout the study (at baseline, after 1 and 3 weeks of treatment)

	Sham (n = 8)	CRF (n = 7)	CRF100 (n = 6)	CRF200 (n = 7)	CRF400 (n = 10)	CRF600 (n = 7)
RBC (x 10^{12}/L)						
0w	7.41±0.07	7.43±0.09	7.03±0.12	7.53±0.11	7.60±0.09	7.03±0.07
1w	7.11±0.08	6.11±0.12***a***	7.02±0.21***b***	6.80±0.14***b***	7.62±0.06***abd***	8.08±0.11***abcd***
3w	7.77±0.13	6.41±0.11***a***	7.92±0.40***b***	7.48±0.17***b***	8.39±0.21***bd***	10.53±0.20***abcde***
Hb (g/dL)						
0w	13.85±0.13	13.63±0.18	13.55±0.13	13.54±0.17	13.68±0.13	13.66±0.18
1w	13.94±0.11	12.26±0.22***a***	13.58±0.37***b***	13.86±0.20***b***	14.55±0.14***b***	16.33±0.29***abcde***
3w	14.07±0.15	12.06±0.18***a***	14.65±0.52***b***	13.89±0.32***b***	15.45±0.37***abd***	20.08±0.37***abcde***
Ht (%)						
0w	41.12±0.54	41.34±0.59	38.40±0.31	40.90±0.55	41.25±0.45	39.23±0.41
1w	38.02±0.20	32.13±0.63***a***	38.73±1.19***b***	36.21±0.92***b***	41.88±0.49***abcd***	50.11±0.93***abcde***
3w	41.77±0.80	33.56±0.57***a***	43.37±2.09***b***	38.03±0.87***b***	46.54±1.43***bd***	66.16±1.16***abcde***
PLT (x 10^{9}/µL)						
0w	687.25±25.87	681.43±14.27	786.17±44.49	727.57±29.97	719.60±16.77	758.43±20.73
1w	722.62±27.95	867.71±19.32	910.83±77.56	911.00±60.80	790.904±48.17	825.57±36.99
3w	743.62±28.36	888.71±51.18	762.50±62.12	815.43±43.39	632.10±31.82***bd***	400.86±47.98***abcde***
RET (%)						
0w	1.95±0.17	2.47±0.29	1.15±0.13	2.34±0.23	1.99±0.13	1.53±0.27
1w	1.73±0.31	2.18±0.23	3.05±0.27	5.27±0.35***abc***	9.23±0.53***abcd***	6.41±0.53***abce***
3w	1.61±0.19	2.53±0.28	3.30±0.25***a***	2.14±0.21	1.46±0.17***bc***	2.38±0.45
RET (x10^{9}/L)						
0w	144.39±12.38	183.41±20.89	80.37±8.20	177.06±18.25	151.09±10.19	108.08±19.72
1w	122.47±22.47	134.11±15.26	215.92±23.86	358.17±25.05***ab***	701.81±39.67***abcd***	520.08±45.99***abcde***
3w	124.77±14.56	161.67±17.87	258.28±13.61***a***	158.84±14.67	119.90±12.17***c***	252.18±48.78***ae***
RPI						
0w	1.94±0.16	2.47±0.28	1.07±0.10	2.34±0.23	1.99±0.14	1.48±0.26
1w	1.72±0.31	1.34±0.23	3.02±0.44	4.86±0.51***ab***	10.13±0.55***abcd***	8.50±0.80***abcd***
3w	1.60±0.19	1.37±0.15	3.42±0.17***ab***	1.93±0.17	1.57±0.16***c***	3.81±0.74***abde***
EPO (mIU/mL)						
0w	2.48±0.09	3.04±0.22	2.03±0.06	3.23±0.25	2.77±0.31	2.43±0.90
1w	3.52±0.39	5.68±0.41***a***	3.48±0.27***b***	5.44±0.46***ac***	4.32±0.32***bc***	3.65±0.62***b***
3w	4.38±0.53	9.71±1.28***a***	4.31±0.49***b***	4.55±0.26***b***	4.34±0.45***b***	4.80±0.63***b***

Results are presented as mean ± SEM. ***a*** $P<0.05$ vs Sham group; ***b*** $P<0.05$ vs CRF group; ***c*** $P<0.05$ vs CRF100 group; ***d*** $P<0.05$ vs CRF200 group; ***e*** $P<0.05$ vs CRF400 group (Mann-Whitney test). 0w, start of protocol; 1w, 1 week after the start of rHuEPO treatment; 3w, 3 weeks after the start of rHuEPO treatment (end of protocol); CRF, chronic renal failure; EPO, erythropoietin; Hb, haemoglobin; Ht, haematocrit; PLT, platelets; RBC, red blood cells; RET, reticulocytes; RPI, reticulocyte production index.

(Table 1). No other significant changes were observed in the CRF group regarding haematological parameters.

rHuEPO treatment induced a significant increase in RBC count, Hb concentration and Ht values in all CRF groups under rHuEPO therapy, as compared to the CRF group. The highest augmentation was found in the CRF400 and CRF600 groups, in which Hb concentration reached, at the end of protocol, 15.45 ± 0.37 and 20.08 ± 0.37 g/dL, respectively, compared to 12.06 ± 0.18 g/dL in the CRF animals. A low platelet count was found in the CRF400 and CRF600 groups at the end of the protocol. After one week of treatment, reticulocyte count and reticulocyte production index (RPI) were increased in the CRF200, CRF400 and CRF600 groups, returning to values similar to those of the Sham animals at the end of the protocol, excepting for the CRF600 rats whose values remained significantly increased. The CRF100 group presented a significant increase in reticulocyte count and RPI only after 3 weeks of treatment.

While, increased serum EPO levels were found in the CRF group, when compared to the Sham group, unchanged values were encountered in all the CRF groups under rHuEPO treatment (Table 1).

Table 2 Serum biochemical data throughout the study (at baseline, after 1 and 3 weeks of treatment) and urinary biochemical data at the end of protocol

	Sham	CRF	CRF100	CRF200	CRF400	CRF600
			Serum biochemical data			
BUN (mg/dL)						
0w	22.54±0.83	22.71±0.87	25.58±0.83	24.07±0.77	24.16±0.73	24.77±0.48
1w	23.07±0.64	56.33±2.40***a***	63.50±2.36***a***	56.78±2.41***a***	60.49±3.20***a***	62.88±2.39***a***
3w	22.64±0.91	54.10±3.63***a***	53.27±7.77***a***	56.63±1.97***a***	60.01±3.05***a***	58.08±1.69***a***
Creatinine (mg/dL)						
0w	0.37±0.01	0.35±0.01	0.42±0.02	0.39±0.01	0.37±0.01	0.36±0.02
1w	0.33±0.01	0.99±0.08***a***	1.18±0.06***a***	1.34±0.16***a***	1.23±0.10***a***	1.17±0.08***a***
3w	0.45±0.04	1.00±0.07***a***	1.26±0.08***a***	1.27±0.23***a***	1.11±0.09***a***	1.23±0.12***a***
CRP (U/L)						
0w	538.49±39.25	552.72±37.64	447.52±20.33	555.59±30.32	556.80±46.46	583.59±52.07
1w	687.91±40.71	592.39±52.52	859.51±35.17***ab***	705.95±49.94	637.29±30.70***c***	691.28±50.34***c***
3w	575.03±33.24	820.61±48.02***a***	767.56±27.10***a***	771.54±64.15***a***	605.91±42.61***b***	551.63±34.25***bcd***
			Urinary biochemical data			
BUN (mg/dL)	6573.00±658.14	2236.67±156.76***a***	2464.83±157.73***a***	2020.75±329.91***a***	2166.00±157.67***a***	2098.00±161.05***a***
Creatinine (mg/dL)	116.99±13.72	37.32±2.40***a***	45.40±6.97***a***	31.15±4.89***a***	34.11±2.24***a***	38.44±3.18***a***
AE (mg/L)	3.25±0.25	31.40±7.81***a***	9.33±2.15a***b***	7.25±1.89a***b***	19.43±2.33***acd***	28.33±5.88***acd***
CC (mL/h/rat)	87.66±5.59	40.33±2.95***a***	38.23±4.72***a***	31.45±6.19***a***	37.21±5.66***a***	34.11±3.02***a***
BUN C (mL/h/rat)	97.08±4.48	43.90±3.87***a***	54.32±6.74***a***	39.70±7.22***a***	43.96±6.81***a***	36.88±3.56***a***
GFR (mL/h/rat)	91.83±3.93	42.06±3.37***a***	48.38±5.10***a***	35.26±6.56***a***	40.38±6.14***a***	35.46±3.26***a***

Results are presented as mean ± SEM. ***a*** *P*<0.05 vs Sham group; ***b*** *P*<0.05 vs CRF group; ***c*** *P*<0.05 vs CRF100 group; ***d*** *P*<0.05 vs CRF200 group (Mann-Whitney test). 0w, start of protocol; 1w, 1 week after the start of rHuEPO treatment; 3w, 3 weeks after the start of rHuEPO treatment (end of protocol); AE, Albumin excretion; BUN C, blood urea nitrogen clearance; CC, Creatinine Clearance; CRF, Chronic renal failure; GFR, Glomerular filtration rate

Regarding serum biochemical data (Table 2), one week after rHuEPO treatment, serum blood urea nitrogen (BUN) and creatinine concentrations significantly increased in all CRF groups (with or without rHuEPO treatment), *versus* the Sham group, without differences between groups; the values remained significantly increased until the end of the protocol. At that moment, serum C-reactive protein (CRP) concentration was also significantly increased in the CRF group, when compared to the Sham, CRF400 and CRF600 groups. Concerning the biochemical urinary markers (Table 2), all CRF groups (with and without treatment), compared to the Sham, presented significantly decreased urinary BUN and creatinine levels, and clearances, as well as reduced glomerular filtration rates (GFR), without differences between groups. Albumin excretion was significantly increased in CRF, CRF400 and CRF600 groups, when compared to Sham, CRF100 and CRF200 groups. CRF100 and CRF200 groups presented also significantly increased albumin excretion values, *versus* the Sham group.

Blood pressure and body and tissues weights

At the end of the protocol, a significantly reduced BW and a significant increased relative kidney weight (KW/BW) were observed in all CRF groups (with and without rHuEPO treatment), when compared to the Sham group (Table 3). In addition, the relative spleen weight (SW/BW) was significantly higher in the CRF600 group, as compared to Sham and to the other CRF groups, except to the CRF100 group.

Heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) values were significantly increased in the CRF group, *versus* the Sham and the CRF groups under rHuEPO treatment (Table 3).

All the rHuEPO-treated CRF groups presented a significant reduction in HR, SBP, DBP and MBP

Table 3 Body weight, tissues weight and arterial blood pressure, at the end of protocol

	Sham	CRF	CRF100	CRF200	CRF400	CRF600
BW(kg)	0.420±0.007	0.389±0.007***a***	0.379±0.004***a***	0.369±0.010***a***	0.369±0.007***a***	0.367±0.006***a***
Organ/BW(g/kg)						
KW/BW	2.767±0.071	3.827±0.185***a***	3.799±0.401***a***	3.441±0.167***a***	3.508±0.128***a***	3.509±0.163***a***
SW/BW	1.869±0.130	2.334±0.112	2.555±0.181***a***	2.073±0.162	2.368±0.108	3.138±0.214 ***abde***
Arterial blood pressure						
HR (Beat/min)	334.43±3.18	411.88±3.39***a***	397.68±2.70***ab***	372.41±7.35***abc***	380.56±5.34***ab***	368.52±3.99***abc***
SBP (mmHg)	106.25±0.70	190.75±1.64***a***	127.24±0.70***ab***	141.75±1.55***abc***	137.45±1.32***abc***	150.44±1.24***abcde***
DBP (mmHg)	90.18±1.47	150.38±1.58***a***	109.29±1.15***ab***	109.25±1.16***ab***	102.40±3.76***ab***	117.00±1.64***abcde***
MBP (mmHg)	91.62±2.31	152.63±1.43***a***	114.20±1.80***ab***	122.00±1.99***abc***	114.20±1.88***ab***	133.80±1.11***abcde***

Results are presented as mean ± SEM. ***a*** *P*<0.05 vs Sham group; ***b*** *P*<0.05 vs CRF group; ***c*** *P*<0.05 vs CRF100 group; ***d*** *P*<0.05 vs CRF200 group; ***e*** *P*<0.05 vs CRF400 group; ***f*** *P*<0.05 vs CRF600 group (Mann-Whitney test). BW, body weight; DBP, diastolic blood pressure; HR, heart rate; K, kidney; MBP - mean blood pressure; SBP, systolic blood pressure; SW, spleen weight; W, weight.

Table 4 Total score of advanced and mild kidneys lesions of rat groups under study

	Sham	CRF	CRF100	CRF200	CRF400	CRF600
Advanced glomerular lesions						
Thickening of GBM	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mesangial expansion	0	0.00 ± 0.00	1.00 ± 0.00***ab***	0.00 ± 0.00***c***	0.40 ± 0.22***c***	0.00 ± 0.00***c***
Nodular sclerosis	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.13	0.43 ± 0.43
Global Glomerulosclerosis	0	3.00 ± 0.00***a***	0.00 ± 0.00***b***	0.43 ± 0.43***b***	0.30 ± 0.30***b***	0.00 ± 0.00***b***
Mild glomerular lesions						
Glomerular atrophy	0	3.00 ± 0.00***a***	1.33 ± 0.21***ab***	1.57 ± 0.37***ab***	0.50 ± 0.22***abcd***	0.28 ± 0.18***bcd***
Hypercellularity	0	0.00 ± 0.00	1.00 ± 0.00***ab***	0.43 ± 0.20***ac***	0.60 ± 0.16***ab***	1.00 ± 0.00***abd***
Dilatation of BS	0	0.00 ± 0.00	1.17 ± 0.40***ab***	0.43 ± 0.20***a***	0.20 ± 0.13	0.28 ± 0.18***c***
Advanced tubulointerstitial lesions						
Hyaline cylinders	0	2.86 ± 0.14***a***	1.00 ± 0.36***ab***	1.71 ± 0.28***ab***	0.90 ± 0.23***ab***	0.14 ± 0.14***bcde***
Granular cylinders	0	2.86 ± 0.14***a***	0.67 ± 0.21***ab***	0.71 ± 0.18***ab***	0.50 ± 0.17***ab***	0.14 ± 0.14***bd***
Tubular calcification	0	3.00 ± 0.00***a***	0.00 ± 0.00***b***	0.00 ± 0.00***b***	0.00 ± 0.00***b***	0.00 ± 0.00***b***
Necrosis	0	2.14 ± 0.14***a***	0.00 ± 0.00***b***	0.00 ± 0.00***b***	0.00 ± 0.00***b***	0.00 ± 0.00***b***
IFTA	0	3.00 ± 0.00***a***	0.50 ± 0.22***ab***	0.28 ± 0.28***b***	0.00 ± 0.00***bc***	0.00 ± 0.00***bc***
Mild tubulointerstitial lesions						
Tubular dilatation	0	1.86 ± 0.34***a***	1.67 ± 0.21***a***	1.71 ± 0.36***a***	1.30 ± 0.26***a***	1.28 ± 0.36***a***
Inters. inflammat. infilt.	0	2.00 ± 0.00***a***	1.00 ± 0.00***ab***	1.28 ± 0.36***a***	1.30 ± 0.15***ab***	0.14 ± 0.14***bcde***
Hidropic tubular degen.	0	----	3.00 ± 0.00***a***	1.14 ± 0.39***ac***	1.60 ± 0.22***ac***	1.00 ± 0.00***ace***
Vacuolar tubulardegen.	0	----	1.00 ± 0.00***a***	0.00 ± 0.00***c***	0.00 ± 0.00***c***	0.00 ± 0.00***c***
Vascular lesions						
Arteriolosclerosis	0	2.00 ± 0.00***a***	0.83 ± 0.17***ab***	0.28 ± 0.28***b***	0.20 ± 0.20***bc***	0.00 ± 0.00***bc***
Arteriosclerosis	0	1.00 ± 0.00***a***	1.00 ± 0.00***a***	0.57 ± 0.30***a***	0.60 ± 0.16***a***	1.00 ± 1.00***a***

Results are presented as mean ± SEM. ***a*** *P*<0.05 vs Sham group; ***b*** *P*<0.05 vs CRF group; ***c*** *P*<0.05 vs CRF100 group; ***d*** *P*<0.05 vs CRF200 group; ***e*** *P*<0.05 vs CRF400 group (Mann-Whitney test). BS, Bowman's Space; Degen., Degeneration; GBM, glomerular basement membrane; IFTA, interstitial fibrosis and tubular atrophy; Inters. Inflammat. Infilt., Interstitial inflammatory infiltrate.

when compared to the untreated CRF group; however, a trend towards increased SBP, DBP and MBP and decreased HR was found, accompanying rHuEPO dose increment.

Histopathological findings

At the end of protocol, no abnormal features were found in the kidneys of the Sham animals (Table 4). Regarding the CRF group, several glomerular and tubulointerstitial lesions were found: all the rats (n = 7) presented global glomerulosclerosis (Fig. 1a2; Table 4) and glomerular atrophy (Fig. 1b2; Table 4), with a significantly increased total score for both advanced (Fig. 1a1) and mild (Fig. 1b1) glomerular lesions, when compared to all the other groups. Tubular calcification and necrosis were present in the CRF animals' kidneys (Table 4), but absent in the rHuEPO-treated CRF groups. In addition, the CRF group presented hyaline (Fig.1c2) and granular cylinders (Table 4), as well as, interstitial fibrosis and tubular atrophy (IFTA) (Fig. 1c3, Table 4), that were reduced or even absent in the rHuEPO-treated CRF groups. Interstitial inflammatory infiltrate (Fig. 1d2) and tubular dilations (Fig. 1d3) were also observed in the CRF group (Table 4). When necrosis is present, hidropic or vacuolar tubular degeneration is no longer observed and, therefore, these lesions were not quantified in the CRF group. The total score for both advanced (Fig. 1c1) and mild (Fig. 1d1) tubulointerstitial lesions were significantly higher in the CRF group, compared to all the other groups. Pyogranulomas were found in all CRF rats, a feature that was not found in the CRF animals under rHuEPO treatment. A higher score for arteriolosclerosis (Fig. 1e2) was observed in the CRF group. Arteriosclerosis score (Fig. 1e3) was similar or decreased in the rHuEPO-treated CRF groups, as compared to the untreated CRF group.

The CRF100 group presented the highest total score for advanced glomerular lesions (Fig. 1a1), compared to all the other CFR groups under rHuEPO therapy. In addition, all the rats of the CRF100 group (6/6) presented mesangial expansion, hypercellularity (Fig. 1a3), dilatation of the Bowman's Space (Fig. 1b3) and glomerular atrophy (Table 4). Advanced tubulointerstitial lesions, including hyaline (4/6) and granular cylinders (4/6), and some degree of IFTA (3/6), were found in the CRF100 rats (Table 4). Regarding the mild tubulointerstitial lesions, vacuolar tubular degeneration (6/6) was found only in the CRF100 group; hidropic tubular degeneration (6/6), tubular dilation (6/6) and interstitial inflammatory infiltrate (6/6) were also observed in this group (Table 4). The CRF100 group presented also arteriolosclerosis (5/6) and arteriosclerosis (6/6) (Table 4).

Regarding the other CRF groups under rHuEPO treatment (CRF200, CRF400 and CRF600), lower total scores for advanced (Fig. 1a1) and mild (Fig. 1b1) glomerular lesions were found, as compared to the untreated CRF group. Glomerular atrophy was particularly high in CRF200 group (6/7), as well as, hypercellularity in the CRF200 (3/7), CRF400 (6/10) and CRF600 (7/7) groups (Table 4). Hidropic tubular degeneration (7/7, 10/10, 7/7, respectively) and tubular dilation (6/7, 9/10, 6/7, respectively) were present in the CRF200, CRF400 and CRF600 groups, along with interstitial inflammatory infiltrate in the CRF200 (5/7) and CRF400 (10/10) groups (Table 4). Arteriosclerosis was also found in the CRF200 (3/7), CRF400 (6/10) and CRF600 (7/7) groups (Table 4).

Adjacent to the outer surface of the scar in the 5/6 remnant kidney of the rHuEPO-treated CRF rats, dysfunctional renal tissue was found, presenting global glomerulosclerosis, tubular dilation and, in some rats, hyaline and granular cylinders, tubular

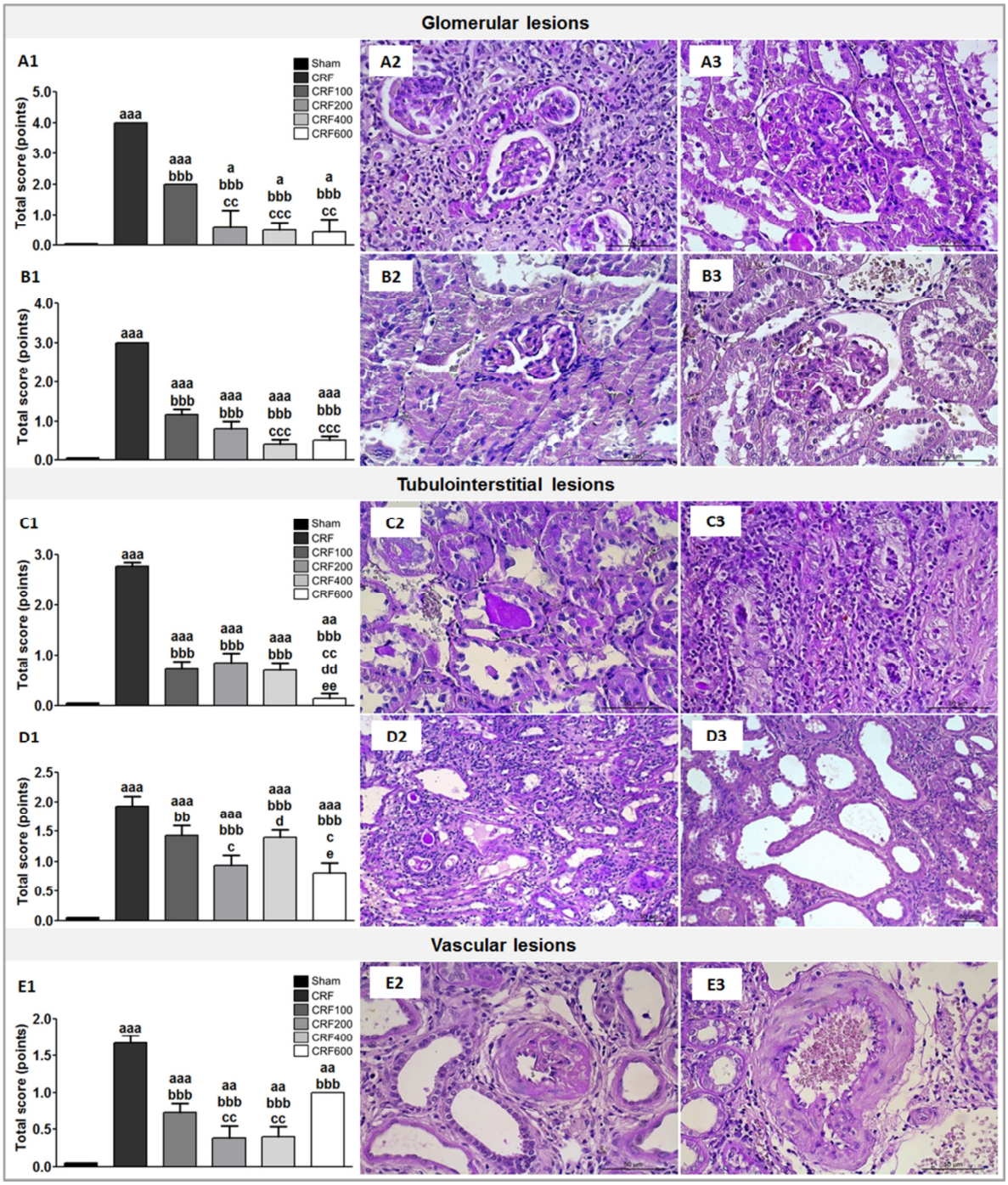

Fig. 1 Glomerular, tubulointerstitial and vascular lesions. Total score and representative lesions observed in kidneys of rat groups under study, at the final time (PAS staining): A1, total score for advanced glomerular lesions; A2, glomerulosclerosis; A3, mesangial expansion and glomerular hypercellularity; B1, total score of mild glomerular lesions; B2, glomerular atrophy; B3, dilatation of the Bowman's Space; C1, total score for advanced tubulointerstitial lesions; C2, hyaline cylinder; C3, interstitial fibrosis and tubular atrophy (IFTA); D1, total score for mild tubulointerstitial lesions; D2, interstitial inflammatory infiltrate; D3, tubular dilatations; E1, total score for vascular lesions; E2, arteriolosclerosis; E3, arteriosclerosis. Results are presented as mean ± standard error of the mean (SEM): a $P < 0.05$, aa $P < 0.01$, aaa $P < 0.001$ vs Sham group; bb $P < 0.01$, bbb $P < 0.001$ vs CRF group; c $P < 0.05$, cc $P < 0.01$, ccc $P < 0.001$ vs CRF100 group; d $P < 0.05$, dd $P < 0.01$ vs CRF200 group; e $P < 0.05$, dd $P < 0.01$ vs CRF400 group (Mann-Whitney Test).

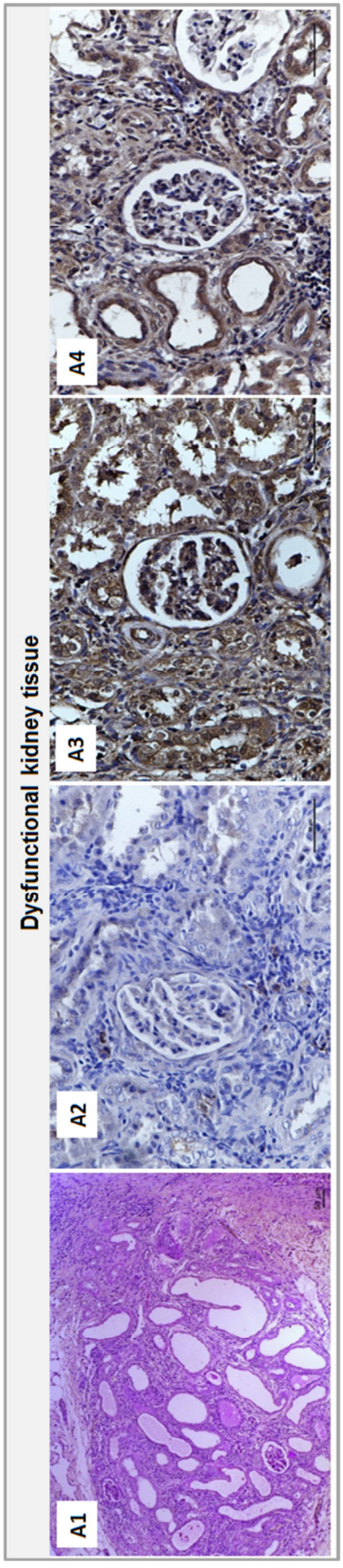

Fig. 2 Kidney dysfunctional areas and immunostaining for several proteins. A1, representative tissue for kidney dysfunctional areas (PAS staining). A2, erythropoietin immunostaining: negative reaction; A3, NF-κB immunostaining: intense positive nuclear immunoreactivity in glomerulus and convulated tubules (CT); A4, TGFβ1 immunostaining: intense positive cytoplasmic immunoreactivity in CT.

necrosis and IFTA (Fig. 2A1). These areas were negative in the immunostaining for EPO protein (Fig. 2A2) and strongly positive for nuclear factor kappa B (NF-κB) (Fig. 2A3) and transforming growth factor beta 1 (TGFβ1) (Fig. 2A4).

The mild tubulointerstitial lesions observed in all CRF rats (without and with treatment) were significantly and negatively correlated with GFR ($r = –0.283$, $P = 0.003$) and positively with serum creatinine ($r = 0.293$, $P = 0.001$); no correlations were found between glomerular lesions and renal function data.

Renal and liver protein expression of HIF-2α, EPO and EPOR

A significant reduction in renal tissue expression of hypoxia inducible factor 2 alpha (HIF-2α) and EPO was observed in CRF and CRF100 groups, when compared to the Sham group (Fig. 3a1 – 3a3), and no protein expression of both HIF-2α and EPO was found in the other CRF groups (CRF200, CRF400 and CRF600; Fig. 3a1 and 3a4).

Significantly increased liver HIF-2α and EPOR protein expression was found in the CRF and CRF600 groups, when compared to the Sham group (Fig. 3c,d). Increased EPO protein expression levels were only found in the liver of CRF rats, as compared to the Sham animals (Fig. 3e).

Renal expression of inflammatory and fibrosis markers

Regarding the renal cortex and medulla expression of NF-κB, the CRF group presented a higher nuclear expression of NF-κB, when compared to the Sham group (Fig. 4a1–4a3 and 4b1–4b3). The rHuEPO-treated CRF groups presented a trend towards a dose-dependent reduction in the NF-κB protein levels (Fig. 4a1 and 4a4; 4b1 and 4b4).

TGFβ1 expression assessed in the renal cortex (Fig. 4c1–4c4) and medulla (Fig. 4d1), as well as in

areas of renal fibrosis (Fig. 4e1–4e3), was particularly increased in the CRF group, when compared to the Sham group (Fig. 4c1; 4d1; 4e1; Table 4). Regarding the rHuEPO-treated CRF groups, a significant reduction of TGFβ1 scores was found in the renal cortex and in the fibrosis areas of the CRF200, CRF400 and CRF600 groups, when compared to the CRF group, achieving values similar to those found in the Sham group (Fig. 4c1 and 4e1). The CRF100 group presented a TGFβ1 score, in the fibrosis areas, similar to that found for the CRF group (Fig. 4e1). In the renal medulla, all the rHuEPO-treated CRF groups presented significantly reduced TGFβ1 scores, when compared to the CRF group, without differences between groups (Fig. 4d1).

Kidney mRNA levels of interleukin (IL)-6 were significantly increased in the CRF group, when compared to the Sham group. The CRF groups under rHuEPO treatment presented a significant down-regulation of IL-6 mRNA levels, compared to Sham and CRF groups (Fig. 4f).

In addition, while IL-1β overexpression was found in the CRF and CRF100 groups, when compared to the Sham, the CRF200, CRF400 and CRF600 groups presented a significantly lower IL-1β expression (Fig. 4g).

DISCUSSION

After 3 weeks of nephrectomy, the CRF rats developed a moderate degree of renal insufficiency, along with glomerulosclerosis and tubulointerstitial lesions. The substantial surgical removal of nephrons induced a compensatory hypertrophy of the remnant kidney, as viewed by the increased KW/BW ratio of these rats. In addition, the CRF rats developed a moderate anaemia; even though serum EPO levels were increased when compared to those of the Sham and rHuEPO-treated CRF groups. Indeed, it has been reported that in both CKD patients and animal models of CKD, the serum EPO levels are within the normal range or even increased.[20–24] It is suggested that the serum EPO levels in CRF rats are, probably, insufficient to overcome renal anaemia.[22, 25, 26] The remnant kidney of the CRF animals maintained some renal capacity to induce EPO synthesis, as showed by renal HIF-2α and EPO protein expression; even though, their expression levels were clearly reduced, when compared to the Sham kidneys, as the activation of HIF-2α appears to reduce with the progression of CKD.[27] Serum CRP levels, as well as kidney inflammatory markers (IL-6, IL-1β and NF-κB), were increased in CRF group and might contribute to reduce renal EPO production.[28–30] Although, the kidney is the major site of EPO production in adults, in conditions of renal injury and hypoxia, the liver may increase EPO synthesis.[31, 32] As occurs in the kidney tissue,[33, 34] HIF-2α mediates EPO production in the liver.[35, 36] In our study, the CRF group presented an increased liver HIF-2α expression, when compared to the Sham group, accompanied by overexpression of EPO and EPOR, thus suggesting an increased contribution of the liver tissue to the serum EPO levels, as reported by others.[37, 38]

Inflammation, another common feature of CKD patients[39, 40] can induce functional iron deficiency due to its stimulatory effect on hepcidin synthesis,[41] which is the major regulator of iron homeostasis. In the current study, no alterations, eventually evoked by inflammation, were observed in iron metabolism that could influence erythropoiesis (data not shown).

TGFβ1a well known pro-fibrotic factor,[42] was found to be overexpressed in the kidney of CRF rats, suggesting a major contribution to the fibrosis and glomerulosclerosis observed and to the increased expression of pro-inflammatory cytokines (Il-6 and IL-1β), thus aggravating renal injury.

The CRF groups under rHuEPO treatment presented correction of anaemia, thus showing a

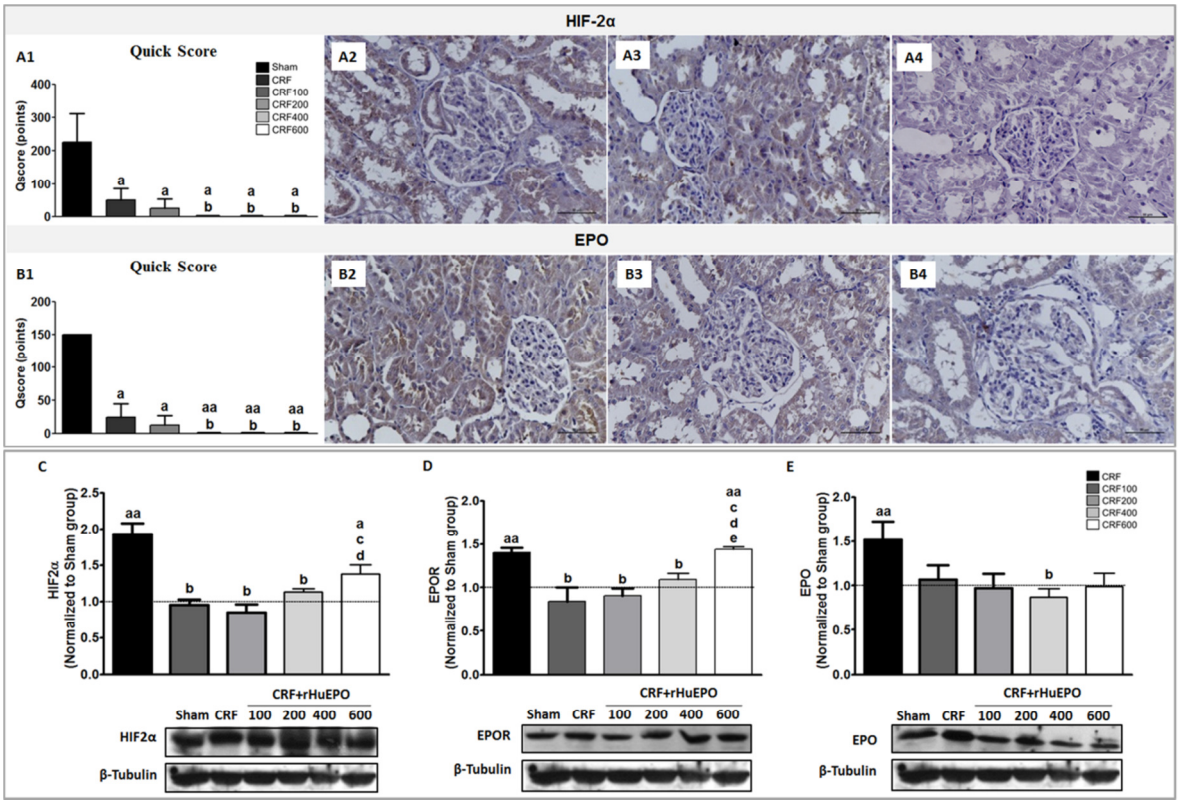

Fig. 3 Immunostaining for hypoxia inducible factor 2 alfa (HIF-2α) and erythropoietin (EPO) in kidney. HIF-2α and EPO were not expressed in the glomerulus or renal medulla tubules (negative reaction). A1, quick score for HIF-2α; A2, HIF-2α immunostaining Sham group: moderate positive nuclear and cytoplasmic immunoreactivity in convulated tubules (CT); A3, HIF-2α immunostaining CRF and CRF100 groups: light positive nuclear and cytoplasmic immunoreactivity in CT;A4, HIF-2α immunostaining CRF200, CRF400 and CRF600 groups: negative reaction; B1, quick score for EPO; B2, EPO immunostaining Sham group: moderate positive cytoplasmic immunoreactivity in CT; B3, EPO immunostaining CRF and CRF100 groups: light positive cytoplasmatic immunoreactivity in CT; B4, EPO immunostaining CRF200, CRF400 and CRF600 groups: negative reaction. Evaluation of liver proteins by Western blotting and representative image of Western blot for the different groups. C, HIF-2α; D, EPO receptor (EPOR); E, EPO. Results are presented as mean ± standard error of the mean (SEM): a $P < 0.05$, aa $P < 0.01$ vs Sham group; b $P < 0.05$ vs CRF group; c $P < 0.05$ vs CRF100 group; d $P < 0.05$ vs CRF200 group; e $P < 0.05$ vs CRF400 group (Mann-Whitney test).

clear response to the increasing rHuEPO stimuli (Table 1). The CRF groups treated with highest rHuEPO doses (CRF400 and CRF600) presented a reduction in platelet count at the end of protocol, which was more pronounced in the CRF600 group. This reduction in platelet count can result from competition between precursor cells of the erythroid and megakaryocytic cell lines that share a common precursor.[43] The increased SW/BW ratio suggests that the platelet reduction might also result from sequestration of platelets in the spleen. Indeed, this group (CRF600) presented the highest Ht, and, therefore, increased blood viscosity, which favours splenic stasis and cell sequestration. Another possible explanation is that this high rHuEPO

stimuli triggers extramedullary erythropoiesis (data not shown).

The Hb and Ht values observed in CRF400 and CRF600 groups are above those recommended for CKD patients;[44] however, the impact of high Hb levels and Ht are still debatable, as naturally occurring Hb levels >12 g/dL are not associated with increased mortality in HD patients, suggesting that high Hb are not always harmful in CKD patients.[45] Until recently, higher ESA doses were used to overcome the hyporesponse to rHuEPO therapy; this clinical practice was recently changed,[44] as it was associated with a poor outcome of CKD patients.[15, 46] In fact, there is a lack of studies assessing the effects of increasing rHuEPO doses on renal disease progression. All the rHuEPO-treated CRF groups presented an improvement of glomerular and advanced tubulointerstitial renal lesions, as well as in inflammation and fibrosis, these effects were, more pronounced for the highest doses (CRF400 and CRF600). However, all the treated groups presented mild tubulointerstitial lesions that could explain the absence of renal function improvement. Indeed, mild tubular lesions correlated significantly and negatively with GFR and positively with serum creatinine, in agreement with other studies.[47] Kidney histopathological analysis of rHuEPO-treated rats revealed the presence of a dysfunctional tissue, adjacent to the scar areas which can contribute to hamper GFR improvement.

Hypertension is a common cause of CKD and a recognized side effect of rHuEPO therapy. In the current study, all CRF groups developed a hypertensive state that was lower in those under rHuEPO therapy; however, rHuEPO treatment induced a blood pressure rise in a dose-dependent manner. The reduction of blood pressure in the context of CKD may present benefits, as it reduces albumin excretion, thus limiting the progression of CKD and the risk of cardiovascular disease.[48] As observed by others,[9, 49] we found that high blood pressure is associated with increased albumin urinary excretion, but without alterations in other markers of renal function (Table 2). The CRF600 group presented the highest increase in blood pressure, as compared with the other rHuEPO-treated CRF groups, probably due to the increased blood viscosity. This increase in blood pressure is accompanied by worsening of arteriosclerosis, which results from increased Ht and hyperviscosity, thus leading to increased vascular resistance, a stimulus for vascular smooth muscle cell proliferation. In opposition with some studies,[9, 49] no worsening of glomerular or tubulointerstitial lesions was observed. This different outcome may result from the earlier use of a short-term (3 weeks) rHuEPO treatment, in opposition to Garcia *et al.*[9] that evaluated a 6 week treatment without earlier initiation of rHuEPO therapy. The reduced time for the development of a hypertensive state in our study, considering the initiation of rHuEPO treatment one week after nephrectomy, contrasts with the study of Lebel *et al.*,[49] in which rHuEPO therapy started 3 weeks after nephrectomy.

ESA therapy in the clinical practice is usually initiated in a more severe degree of anaemia; however, depending on patients' characteristics and on the evaluation of risk-benefit, the use of this therapy in a moderate degree of anaemia might be viewed as a valid option if the beneficial effects here reported on renal function, fibrosis and inflammation could be further confirmed in the clinical setting.

In conclusion, the model of CKD induced by nephrectomy used in this study showed that the liver may replace the kidney to produce EPO, in conditions of renal anaemia/hypoxia; kidney fibrosis and inflammation seem to be linked with the reduced EPO synthesis and anaemia/hypoxia. Our data also suggest that the crosstalk between

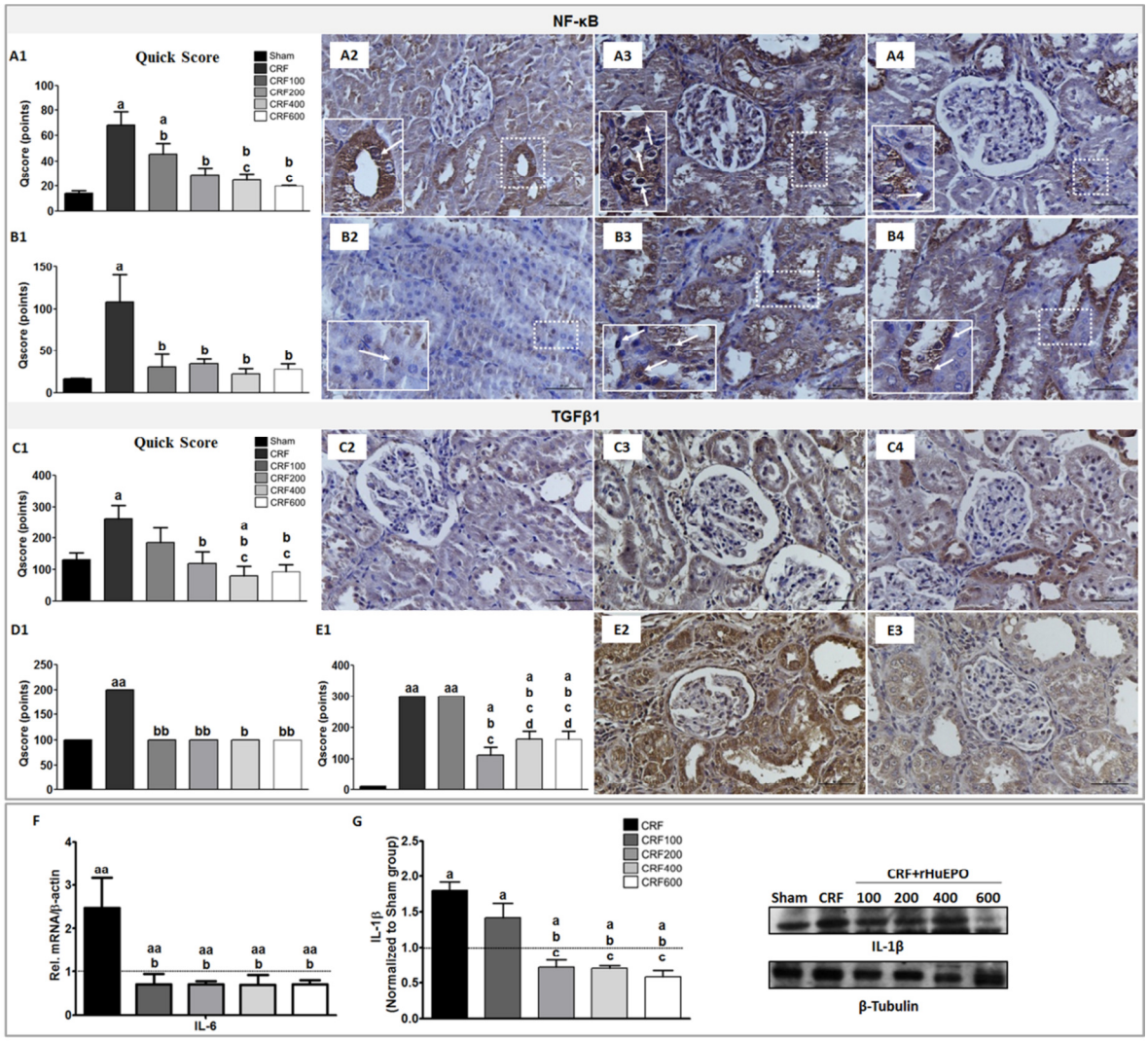

Fig. 4 Immunostaining of NF-κB and TGFβ1 in renal cortex and medulla. For NF-κB only nuclear staining were considered for analysis. A1, quick score for NF-κB in renal cortex; A2, NF-κB immunostaining Sham group: moderate positive nuclear immunoreactivity in convulated tubules (CT); A3, NF-κB immunostaining CRF and CRF100 groups: intense positive nuclear immunoreactivity in glomerulus and CT; A4, NF-κB immunostaining CRF200, CRF400 and CRF600 groups: moderate positive nuclear immunoreactivity in CT; B1, quick score for NF-κB in renal medulla; B2, NF-κB immunostaining Sham group: moderate positive nuclear immunoreactivity in renal medulla tubules; B3, NF-κB immunostaining CRF and CRF100 groups: intense positive nuclear immunoreactivity in renal medulla tubules; B4, NF-κB immunostaining CRF200, CRF400 and CRF600 groups: moderate positive nuclear immunoreactivity interstitial cells; C1, quick score for TGFβ1 in renal cortex; C2, TGFβ1 immunostaining Sham group: light positive cytoplasmic immunoreactivity in CT; C3, TGFβ1 immunostaining CRF and CRF100 groups: intense positive cytoplasmic immunoreactivity in CT and interstitial cells; C4, TGFβ1 immunostaining CRF200, CRF400 and CRF600 groups: moderate positive cytoplasmic immunoreactivity in CT; D1, quick score for TGFβ1 in renal medulla; E1, quick score for TGFβ1 in renal fibrosis areas; E2, TGFβ1 immunostaining CRF and CRF100 groups: intense positive cytoplasmic immunoreactivity in glomerulus, CT and interstitial cells; E3, TGFβ1 immunostaining CRF200, CRF400 and CRF600 groups: moderate positive cytoplasmic immunoreactivity in CT and interstitial cells. F, Kidney relative mRNA expression of interleukin 6 (IL6), with β-actin as the reference gene. G, Kidney protein analysis of IL-1β by Western blot at the end of the protocol and representative image of Western blot for the different groups. Results are presented as mean ± standard error of the mean (SEM). a $P < 0.05$, aa $P < 0.01$ vs Sham group; b $P < 0.05$, bb $P < 0.01$ vs CRF group; c $P < 0.05$ vs CRF100 group; d $P < 0.05$ vs CRF200 group (Mann-Whitney test).

hypertension, anaemia, inflammation and rHuEPO dose is crucial to define the renal risk-benefits of this therapy in CKD. Actually, rHuEPO treatment corrected anaemia and improved urinary albumin excretion, particularly at lower doses; in addition, a short-term treatment with higher doses, used to overcome an episode of reduced hyporesponse to rHuEPO therapy, can present benefits by reducing inflammation, without worsening of renal lesions; however, the pro-hypertensive effect during a long-term treatment should be considered, and carefully managed in order to avoid the negative cardiorenal impact.

ANIMALS AND METHODS

Animals and experimental protocol

Male Wistar rats (Charles River Laboratories, Chatillon-sur-Chalaronne, France), 12 weeks old, were maintained in ventilated cages, in an air conditioned room, subjected to 12 hour dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. The rats were randomly divided in six groups: a Sham-operated group, subjected to surgical process but without kidney mass reduction, and five groups with chronic renal failure induced by a two-stage (5/6) nephrectomy, with surgical excision of both poles of the left kidney (2/3 nephrectomy) by left flank incision and, one week later, complete removal of the right kidney through an identical procedure. After another week, while one of the CRF groups remained under vehicle treatment (saline solution; CRF group), the other four groups started subcutaneous (sc) rHuEPO (100, 200, 400 and 600IU/kg BW per week; CRF100, CRF200, CRF400 and CRF600, respectively; NeoRecormon, Roche Pharmaceuticals, Basel, Switzerland) treatment, three times per week, for a further 3 weeks. Animal experiments were conducted according to the European Community Council directives on animal care and to the national authorities.

The animal HR, SBP, DBP and MBP measures were obtained by the tail-cuff method, using a sphygmomanometer (LE5001 Pressure meter, Panlab Havard Apparatus, Barcelona, Spain).

Sample collection

Blood samples were collected at baseline, one week after the start of treatment and at the end of the protocol (3 weeks of treatment), with rats under anaesthesia (intraperitoneal) with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncuture, from the jugular vein, into Vacuette (Greiner Bio-One, Frickenhausen, Germany) tubes without anticoagulant (to obtain serum) or with K3EDTA for haematological and biochemical studies. Aliquots of serum and plasma were immediately stored at –80ºC until assayed.

For collection of 24-hour urine the rats were enclosed, at the end of the protocol, in metabolic cages during 24 hours with free access to laboratory chow and tap water. Afterwards, urine volume and water consumption were recorded. Aliquots of urine were stored at –80ºC.

At the end of the protocol, after blood collection, rats were killed by cervical dislocation; the kidney and the liver were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighed. In order to isolate total RNA, small portions of kidney and liver from each rat, were immersed in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) upon collection and stored at 4°C for 24 hours; afterwards, samples were frozen at –20ºC. For Western blot analysis organs were

immediately frozen with liquid nitrogen and stored at –80ºC.

Biochemical and haematological assays

The RBC count, Hb concentration, Ht and platelets were assessed in whole blood K_3EDTA using an automated blood cell counter (HORIBA ABX, Amadora, Portugal). Reticulocyte count was determined by microscopic counting on blood smears after vital staining with new methylene blue (reticulocyte stain; Sigma-Aldrich).

The RPI was calculated as previously described by Hillman and Finch[50] [(reticulocyte %/maturation index)*(Ht/normal Ht)], where normal Ht was the mean value presented by the Sham group, and the maturation index (maturation time of circulating blood reticulocytes that increase with premature release of reticulocytes from the bone marrow) was 1 for Sham and 1.5 for anaemic groups.

Serum EPO and CRP levels were evaluated by rat specific ELISA kits, according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA and eBioscience, San Diego, CA, USA, respectively).

Serum BUN and creatinine were analyzed through automatic methods and equipment (Hitachi 717 Chemistry Analyzer, Roche Diagnostics, Basel, Switzerland).

The urinary levels of BUN and creatinine were analyzed using automatic methods (Cobas Integra 400Plus, Roche Diagnostics). Creatinine clearance, BUN clearance and GFR were calculated according to Pestel *et al.*[51]

Gene expression analysis

Kidney RNA isolation and integrity control were performed as previously described by the authors.[24] One microgram of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. One nanogram of cDNA was used for gene expression analysis with qPCR using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs for IL-6 gene (*il6* Forward: ATG TTG TTG ACA GCC ACT GC ; Reverse: TTT TCT GAC AGT GCA TCA TCG). qPCR reactions were performed using the following conditions: enzyme activation at 95ºC for 3 minutes; denaturation at 95ºC for 3 seconds; annealing at 58ºC for 30 seconds. Gene expression was normalized to actin beta (Actb), and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

The liver and kidney proteins were extracted using RIPA buffer (NaCl 150mmol/L, Tris-HCl 50mmol/L pH8, Triton X-100 1%, ethylene glycol tetraacetic acid 5mmol/L, deoxycholic acid 0.5%, sodium lauryl sulfate 0.1%) and ultra-sonication. After centrifugation, protein concentration in supernatant was assayed using the bicinchoninic acid (BCA) method (Thermo Scientific, Pierce, IL, USA). Aliquots of the extract containing 50–100 µg of proteins were separated by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked by using 7% non-fat milk in a solution of Tris-buffered salt with Tween-20. The membranes were incubated, overnight at 4ºC, with rabbit polyclonal antibody anti-EPAS-1 (HIF-2α) 1:200, anti-EPO 1:20, anti-EPOR 1:200 (sc-28706, sc-7956 and sc-697, respectively, from Santa Cruz Biotechnoloy, TX, USA) and goat polyclonal antibody anti-IL1β 1:1000 (AF-501-NA, R&D Systems, MN, USA); afterwards, membranes were incubated with anti-goat or anti-rabbit secondary

antibody-conjugated with horseradish peroxide 1:1000 (ab97120, Abcam, Cambridge, UK, and sc-2004 Santa Cruz Biotechnology, Dallas, TX, USA, respectively). Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL; WesternBright, Advansta, CA, USA). The immunoblot analysis was performed by densitometry (Bio1D++ version 99, Vilber Lourmat). To ensure even loading of the samples, membranes were probed with rabbit anti-β-tubulin antibody 1:500 (sc-9104, SantaCruz Biotechnology). The protein concentration found in each sample was normalized for the protein concentration observed in the Sham group.

Histopathological analysis

Samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3-µm thick sections were stained with Periodic acid Shiff (PAS). All samples were examined by light microscopy, using a Microscope Zeiss Axioplan 2 (Carl Zeiss Microscopy, LLC, NY, USA), and images were captured using a digital microscope camera (Leica DFC450; Leica Microsystems, Wetzlar, Germany). Lesions were evaluated in a double-blinded fashion by the pathologist, who evaluated and quantified lesions, according to methods previously described by our group.[24]

Immunohistochemistry analysis

Tissue sections were incubated in xylene and rehydrated via graded ethanol series to water. To retrieve antigen exposure, the samples were treated with 0.01mol/L citrate buffer solution at 95°C, for 45 minutes. The samples were processed for indirect immune detection, using mouse or rabbit specific horseradish peroxidase (HRP)/ diaminobenzidine (DAB) detection immunohistochemistry (IHC) kit (ab80436, Abcam), according to the manufacturer's protocol. Negative controls were included in all series, by omission of the primary antibodies. Polyclonal rabbit antibodies anti-EPAS-1 (HIF-2α) 1:500 (sc-28706), anti-EPO 1:200 (sc-7956), anti-NFκB p65 1:100 (sc-109) and anti-TGFβ1 (sc-146) 1:100 diluted in TBS-Tween 0.05% were used as primary antibodies; tissue sections were incubated with the primary antibodies, overnight at 4°C in a humidified chamber; excess unbound primary antibody was removed by washing with TBS-Tween 0.05% buffer. Tissue sections were counterstained with hematoxylin, dehydrated, and mounted in a non-aqueous media (DPX Mountant for Microscopy, VWR BDH, Prolabo, PA, USA) and examined with a microscope Nikon Eclipse Ci; images were captured using a digital microscope camera (Nikon DS-Ri2). Immunopositivity was scored in accordance to staining intensity (I) and percentage of positive cells or area (P), as previously described.[24]

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). For comparison between groups Mann-Whitney U test was performed. The strength of the association between the variables was estimated by Spearman correlation coefficient. Statistical significance was accepted at $P < 0.05$. Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS) for Windows, version 22.0 (IBM, Armonk, NY, USA).

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Paper II

Liver iron is a major regulator of hepcidin gene expression via BMP/SMAD pathway in a rat model of chronic renal failure under treatment with high rHuEPO doses

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Liver iron is a major regulator of hepcidin gene expression via BMP/SMAD pathway in a rat model of chronic renal failure under treatment with high rHuEPO doses

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ABSTRACT

Hepcidin is the major central regulator of iron metabolism, controlling iron absorption and mobilization. Considering its interaction with several factors that are altered in chronic kidney disease (CKD), particularly in hyporesponsive CKD patients under therapy with high recombinant human erythropoietin (rHuEPO) doses, we aimed to study the impact of increasing rHuEPO doses on the regulation of iron-hepcidin metabolism. We performed blood, cellular and tissue studies, using the remnant kidney rat model of CKD induced by 5/6 nephrectomy, under rHuEPO (100, 200, 400 and 600IU/Kg body weight [BW]/week) treatment during 3 weeks. We found that the rHuEPO stimulus triggers a first wave to achieve correction of anemia, by inhibiting hepcidin synthesis, favouring erythropoiesis and iron absorption; this continuous stimulus enhanced iron absorption leading to iron overload, as showed by the hepatic iron deposits found in rats treated with higher rHuEPO dose that seems to trigger the up-regulation of hepcidin synthesis through the activation of the BMP6/SMAD pathway. Our data suggests that liver iron overload is an important stimulus for hepcidin synthesis, stronger than the inhibitory effect of high rHuEPO doses; moreover, our findings raise the hypothesis that when high inflammation (triggering hepcidin expression) is associated with increased iron stores in hemodialysis patients, hepcidin expression is also up-regulated via BMP6, enhancing hepcidin synthesis, leading, therefore, to worsening of anemia and, eventually, to a hyporesponse/resistance to rHuEPO therapy.

Keywords: BMP6; chronic renal failure; erythropoietin; hepcidin; iron overload.

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1. Introduction

Iron has essential roles in several physiological processes. The majority of iron in the organism comes from the diet and from the internal iron turnover. The dietary iron is absorbed in the reduced form in the duodenum (Fig.1) and then effluxed through the iron exporter protein ferroportin (FPN), also present in the membrane of macrophages and hepatocytes, according to body iron requirements [1]. Iron circulates in plasma bound to transferrin (Tf), and the Tf receptors (TfR) mediate the cellular uptake of Tf-bound iron (Tf-Fe). There are two TfR types, the TfR1, which is expressed ubiquitously and has high affinity for Tf, and the TfR2 mainly present in hepatocytes [2].

Hepcidin, the central player of iron metabolism, is a peptide synthesized in the liver that regulates iron absorption and mobilization from macrophages and hepatocytes, by binding to FPN, the only known iron exporter, promoting its internalization and degradation [3].

Hepcidin synthesis is highly regulated by several factors, including circulating iron and inflammation, which are stimulatory conditions; and hypoxia, erythropoiesis and erythropoietin, which act as inhibitors of hepcidin production (Fig. 1) [4-6]. Iron overload can be sensed by Tf saturation (TSAT), which is considered a sensor for circulating iron or by iron stores in the liver, and both can induce bone morphogenetic protein 6 (BMP6) expression and activate the BMP-SMAD signaling pathway [7]. Some studies suggested that the induction of hepcidin by TfR2/HFE could also involve the MAPK/ERK signaling pathway [8]. In inflammatory conditions, the synthesis of interleukin (IL)-6 is stimulated, which induces hepcidin expression through the activation of STAT3 pathway [9]. Iron metabolism and erythropoiesis are closely linked, as erythropoiesis efficiency depends on iron availability. Indeed, accelerated erythropoiesis is able to inhibit hepcidin synthesis, in order to stimulate iron absorption and mobilization. Under hypoxic conditions, erythropoiesis is stimulated by increased erythropoietin (EPO) production, which is regulated by the hypoxia inducible factor (HIF) system. EPO itself and the HIF system are able to repress hepcidin synthesis [10, 11]; however, recent studies reported that, *in vivo,* these factors indirectly regulate hepcidin synthesis [12-14]. Thus, despite the advances in the last years, the exact mechanisms underlying the regulation of hepcidin synthesis are not yet well understood.

Anemia, a major complication in chronic kidney disease (CKD) patients can be treated with iron supplementation and erythropoiesis stimulating agents (ESA) [15], but 5-10% of them develop resistance to ESA therapy [16], a condition where higher doses are needed to achieve the target hemoglobin (Hb) values [17, 18].

Hepcidin is increased in hemodialysis (HD) patients [18, 19] and it has been reported to be mainly due to inflammatory conditions [20] and to the reduction of urinary excretion of this peptide [21]. Given the close interaction of hepcidin synthesis with several factors that are altered in CKD patients, particularly altered in hyporesponsive CKD patients, we figured as important to study the impact of increasing ESA doses on the regulation of iron-hepcidin metabolism. To accomplish this goal, we performed blood, cellular and tissue studies that cannot be performed in CKD patients, by using the remnant kidney rat model of CKD-associated anemia to understand the relationship between iron-hepcidin metabolism and erythropoiesis induced by increasing recombinant human EPO (rHuEPO) doses.

2. Methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab., Inc., Chatillon-sur-Chalaronne, France), 12 weeks old, were maintained in ventilated cages, in an air conditioned room, subjected to 12 h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. The rats were randomly divided in six groups (6-7 animals each group): Sham operated group and five groups with chronic renal failure (CRF) induced by a two-stage (5/6) nephrectomy, as previously described [22]. One week after nephrectomy, four CRF groups started subcutaneous rHuEPO (NeoRecormon, Roche, Basel, Switzerland) treatment (100, 200, 400 and 600IU/Kg body weight [BW]/week, respectively, CRF100, CRF200, CRF400 and CRF600) for 3 weeks, while the other CRF group remained under vehicle treatment. All animals received human care and animal experiments were conducted according to the European Communities Council Directives on Animal Care. The experiments were approved by the Portuguese Foundation for Science and Technology and the Local Ethics Committee (ORBEA: Organ Responsible for Animal Welfare) of the Faculty of Medicine from the University of Coimbra.

2.2. Sample collection

Blood samples were collected at baseline, one week after starting rHuEPO treatment and at the end of the protocol, with rats under anesthesia as previously described [22]. Aliquots of serum and plasma were immediately stored at -80ºC until assayed.

At the end of the protocol, after blood collection, the rats were sacrificed by cervical dislocation; liver and duodenum were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighted. In order to isolate total RNA, small portions of liver and duodenum, from each rat, were immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) upon collection and stored at -20ºC. For western blot analysis small portions of the organs were immediately frozen with liquid nitrogen and stored at -80ºC.

2.3. Biochemical and hematological assays

Red blood cells count, hematocrit (Ht) and Hb concentration were assessed in whole blood K_3EDTA, using an automated blood cell counter (HORIBA ABX, Amadora, Portugal). Reticulocyte count was measured by microscopic counting on blood smears after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA).

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were analyzed through automatic methods and equipment (Hitachi 717 Chemistry Analyzer, Roche Diagnostics, Basel, Switzerland).

Serum C-reactive protein (CRP) levels were evaluated by a rat specific ELISA kit, according to manufacture instructions (eBioscience, San Diego, CA, USA).

Serum iron and ferritin were analyzed through automatic methods and equipment (ROCHE Integra 400, Roche Diagnostics, Basel, Switzerland). Serum Tf levels were evaluated by rat specific ELISA kit (Transferrin Rat ELISA Kit, abcam, Cambridge, UK). Tf saturation (TSAT) was calculated using the formula (Iron µg/dL*100)/(Tf mg/dL*2).

2.4. Perls' Prussian blue staining

Liver samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3μm thick sections were stained with Perls' Prussian blue stain. Briefly, tissue sections were deparaffinised, hydrated and immersed in a freshly prepared potassium

ferrocyanide solution (10g/L in 0.1M hydrochloric acid) at room temperature. Counterstaining was made with safranin. All samples were examined by light microscopy (Microscope Nikon Eclipse Ci) and images were captured using a digital microscope camera (Nikon DS-Ri2).

2.5. Gene expression analysis by qPCR

Liver and duodenum RNA isolation and integrity control were performed as previously described [22]. One μg of total RNA was reversely transcribed, using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA), according to manufacturer instructions. One ng of cDNA was used for gene expression analysis with qPCR, using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs (Table 1). qPCR reactions were performed using the following conditions: enzyme activation at 95ºC for 3min; denaturation at 95ºC for 3sec; annealing for 30sec (for each pair of primers, the temperature is referred in Table 1). Gene expression was normalized to actin beta (*actb*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Protein analysis by Western Blot

The liver and duodenum proteins were extracted as previously described [22]. Aliquots of the extract, containing 100 μg of proteins, were separated by reducing SDS-PAGE (10 %) and transferred onto nitrocellulose membranes. The blots were blocked by using 7% non-fat milk in a solution of Tris-buffered salt with Tween-20, and then incubated overnight, at 4ºC, with rabbit anti-SLC40A1 (FPN) antibody 1:100 (ab58695), rabbit anti-SMAD1/5/9 1:500 (ab66737), rabbit anti-SMAD4 1:1000 (ab40759, abcam, Cambridge, UK), rabbit anti-phospho-SMAD1/5/9 (#13820, Cell Signaling Technology, MA, USA), goat anti-hepcidin 1:100 (sc-240553), mouse anti-ERK1 1:200 (sc-376852) and rabbit anti-phospho-ERK1/2 (Thr 202/Tyr 204) 1:100 (sc-16982, Santa Cruz Biotechnoloy, Inc, TX, USA); afterwards, they were incubated with anti-goat (ab97120) or with anti-rabbit (sc-2004) secondary antibody-conjugated with horseradish peroxide 1:1000 (abcam, Cambridge, UK; Santa

Table 1 – List of primer sequences and annealing temperatures

Gene	Primer sequences (5' → 3')	Annealing Temperature
tfrc	F: GGGAGCCATTGTCATACACC R: GTCGCAAAGCAGAGTCTTCC	58ºC
tfr2	F: AGCTGGGACGGAGGTGACTT R: TCCAGGCTCACGTACACAACAG	58ºC
hfe2	F: TTCCAATCCTGCCTCTTTGAT R: GGAAAAGGTGCAAGTTCTCCAA	58ºC
hamp	F: GGCAGAAAGCAAGACTGATGAC R: ACAGGAATAAATAATGGGGCG	58ºC
bmp6	F: GGTGGAGTACGACAAGGAGTT R: GTCACAACCCACAGATTGCTA	56ºC
tmprss6	F: AGAAGGTGGATGTGCAACTGATC R: CTTGCCCTTGCGATAACCA	59ºC
hif1a	F: CTCACCATCAGTTACTTAC R: GTCACCATCATCTGTTAG	58ºC
epas1	F: TGACTTCACTCATCCTTGCGACCA R: ATTCATAGGCAGAGCGGCCAAGTA	59ºC
epo	F: TCTGACTGACCGCGTTACTC R: GCCCAGAGGAATCAGTAGCA	59ºC
slc11a2	F: ATAGCAGACGCCCCCATG R:AGGCCCGAAGTAACATCCAA	58ºC
actb	F: TACAGCTTCACCACCACAGC R: AAGGAAGGCTGGAAGAGAGC	57ºC
gapdh	F: TGCCACTCAGAAGACTGGG R: ACGGATACATTGGGGGTAGG	59ºC

F: Forward; R: Reverse; *actb* - beta – actin; *bmp6* - bone morphogenetic protein 6; *epas1* - endothelial PAS domain-containing protein 1; *epo* – erythropoietin; *gapdh* - glyceraldehyde 3-phosphate dehydrogenase; *hamp* – hepcidin; *hfe2* - hemojuvelin; *hif1a* – hypoxia inducible factor 1 alpha; *slc11a2* - divalent metal transporter 1; *tfrc* – transferrin receptor 1; *tfr2* – transferrin receptor 2; *tmprss6* – matriptase-2.

Cruz Biotechnoloy, Inc, TX, USA). Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL; WesternBright, Advansta, CA, USA). The analysis of the immunoblots was performed by densitometry (Bio1D++ version 99, Vilber Lourmat). To ensure even loading of the samples, all immunoblots were probed with rabbit anti-β-tubulin antibody 1:500 (sc-9104, SantaCruz Biotechnology, TX, USA). The protein concentration in each sample was normalized for Sham group.

2.7. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). For comparison between groups, Mann-Whitney U test was performed. Statistical significance was accepted at $p<0.05$. Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS), for Windows, version 22.0 (IBM, Armonk, NY, USA).

3. Results

The CRF group developed anemia, as showed by the reduction in RBC count, Hb concentration and Ht, when compared to Sham group (Table 2). After one week of rHuEPO treatment, anemia was corrected, in a rHuEPO dose-dependent manner, as showed by the increasing values of RBC count, Hb concentration and Ht. Reticulocyte count increased after one week of rHuEPO treatment, as compared to Sham and CRF groups; this rise in reticulocytes was dose-dependent for CRF100, CRF200 and CRF400 groups, showing the CRF600 group a decrease to a value that was still significantly higher. At the end of the protocol, RBC count, Hb concentration and Ht were even more increased than after one week of treatment; however, we found a normal reticulocyte count, excluding CRF100 and CRF600 groups (Table 2).

Significantly high serum CRP levels (Table 2) were found in CRF, CRF100 and CRF200 groups, compared to Sham, CRF400 and CRF600 groups, at the end of the protocol. CRF400 and CRF600 groups presented CRP levels similar to the Sham group. No significant alterations were found in ALT and AST, for all CRF groups, as compared to Sham group (Table2).

At the end of protocol, we found that serum iron concentration was significantly reduced in the CRF600 group, when compared with Sham, CRF and CRF200 groups (Fig. 2A). Ferritin levels were significantly increased in CRF400 and CRF600 groups, as compared to CRF and CRF200 groups (Fig. 2B). Serum Tf levels (Fig. 2C) were significantly reduced and TSAT (Fig. 2D) increased in CRF200, CRF400 and CRF600 groups versus Sham, CRF and CRF100 groups. Liver histological sections stained with Perls' Prussian blue showed iron deposition as hemosiderin in the CRF600 group (Fig. 3).

Liver TfR1 mRNA levels were up-regulated in CRF, CRF200, CRF400 and CRF600 groups, compared to Sham group, whereas mRNA levels were down-regulated in the CRF100 group (Fig. 4A). The same pattern was found for TfR2 mRNA levels (Fig. 4B). Liver hemojuvelin (HJV) mRNA levels did not differ between groups (Fig. 4C). In accordance with lower hepcidin mRNA levels for all CRF groups, except in CRF600 group (Fig. 4D), hepcidin synthesis was lower, reaching significantly lower values in CRF200 and CRF400 groups (Fig.5A); the CRF600 group presented a significantly higher expression and synthesis of hepcidin (Fig. 4D and Fig. 5A, respectively). BMP6 mRNA levels were down-regulated in all CRF groups, excepting CRF600 group that presented a significant up-regulation (Fig. 4E). Matriptase-2 mRNA levels were increased in CRF, CRF200 and

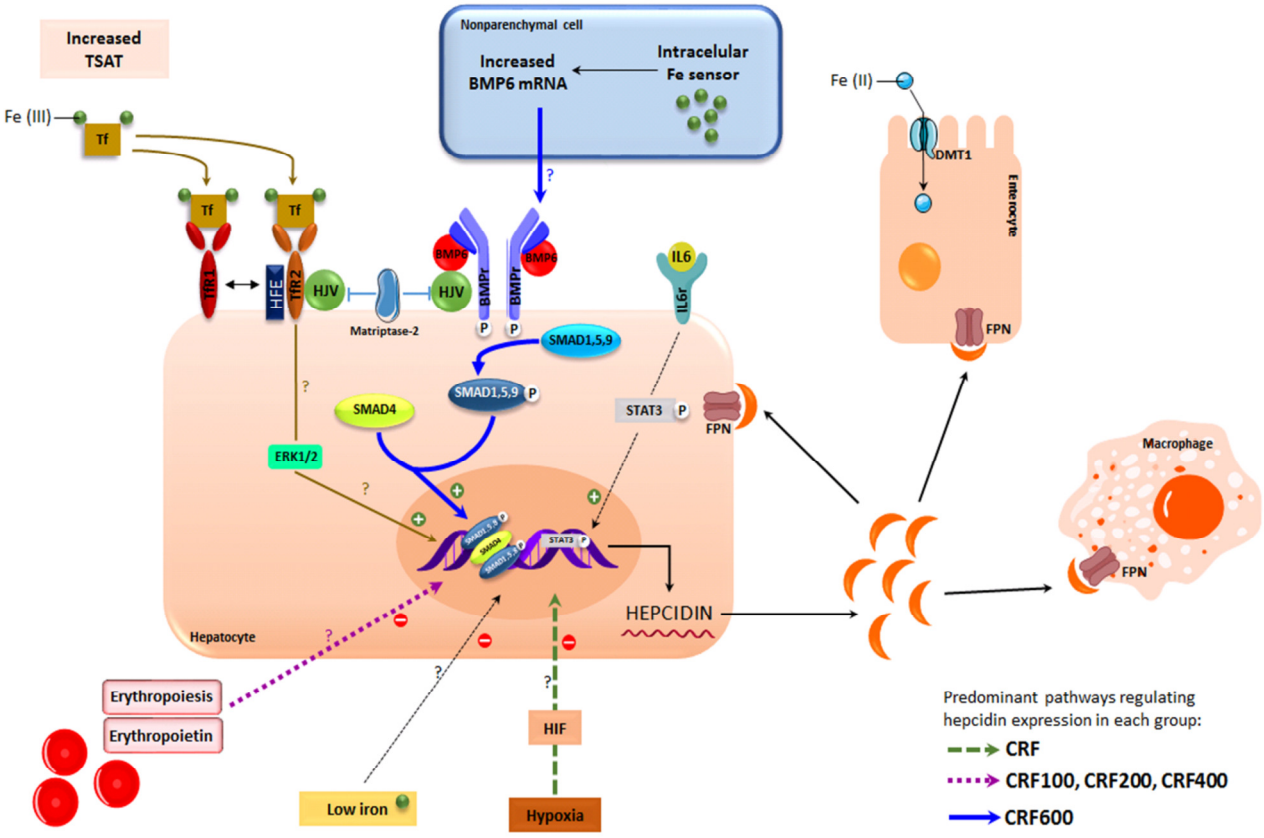

Fig. 1 – Schematic diagram of the molecular pathways and mediators that regulate hepcidin expression in liver. The dietary iron (Fe) is absorbed in the reduced form (Fe II) in the duodenum and transported within the enterocyte through the divalent metal iron transporter 1 (DMT1), and then effluxes, through the iron exporter protein ferroportin (FPN). Hepcidin, the central player of iron metabolism, regulates iron absorption and mobilization from enterocytes, macrophages and hepatocytes, by binding to FPN, an iron exporter, promoting its internalization and degradation. In normal blood iron concentrations, haemochromatosis protein (HFE) and transferrin-bound iron (Tf-Fe) compete for binding to transferrin receptor 1 (TfR1); however, in conditions of increased Tf saturation (TSAT), Tf-Fe displaces HFE that binds to the TfR2 forming a complex with hemojuvelin (HJV) that acts as a co-receptor of bone morphogenetic protein 6 (BMP6), a ligand to BMP receptor, which activates the phosphorylation of SMAD1, SMAD5 and SMAD9, that will form a complex with SMAD4. This complex is translocated into the nucleus, activating (+) the transcription of the hepcidin gene. HJV can be regulated by matriptase-2, which promotes the cleavage of HJV, forming a soluble form that blunts the action of BMP6. Iron stores in the liver that can directly induce BMP6 expression and activate the BMP-SMAD signaling pathway. Induction of hepcidin by TfR2/HFE could also involve the MAPK/ERK signaling pathway. Interleukin (IL)-6 induces hepcidin expression through the activation of STAT3 pathway. Erythropoiesis, erythropoietin, low iron and hypoxia act as inhibitors (-) of hepcidin production.

Table 2 – Hematological and biochemical data at the start of the protocol, 1 and 3 weeks (end of protocol) according to group and rHuEPO treatment

Parameters	Sham	CRF	CRF100	CRF200	CRF400	CRF600
RBC (x 10^{12}/L)						
0w	7.41±0.07	7.43±0.09	7.03±0.12	7.53±0.11	7.60±0.09	7.03±0.07
1w	7.11±0.08	6.11±0.12***a***	7.02±0.21***b***	6.80±0.14***b***	7.62±0.06***abd***	8.08±0.11***abcd***
3w	7.77±0.13	6.41±0.11***a***	7.92±0.40***b***	7.48±0.17***b***	8.39±0.21***bd***	10.53±0.20***abcde***
Hb (g/dL)						
0w	13.85±0.13	13.63±0.18	13.55±0.13	13.54±0.17	13.68±0.13	13.66±0.18
1w	13.94±0.11	12.26±0.22***a***	13.58±0.37***b***	13.86±0.20***b***	14.55±0.14***b***	16.33±0.29***abcde***
3w	14.07±0.15	12.06±0.18***a***	14.65±0.52***b***	13.89±0.32***b***	15.45±0.37***abd***	20.08±0.37***abcde***
Ht (%)						
0w	41.12±0.54	41.34±0.59	38.40±0.31	40.90±0.55	41.25±0.45	39.23±0.41
1w	38.02±0.20	32.13±0.63***a***	38.73±1.19***b***	36.21±0.92***b***	41.88±0.49***abcd***	50.11±0.93***abcde***
3w	41.77±0.80	33.56±0.57***a***	43.37±2.09***b***	38.03±0.87	46.54±1.43***bd***	66.16±1.16***abcde***
Ret (x 10^{9}/L)						
0w	144.39±12.38	183.41±20.89	80.37±8.20	177.06±18.25	151.09±10.19	108.08±19.72
1w	122.47±22.47	134.11±15.26	215.92±23.86	358.17±25.05***ab***	701.81±39.67***abcd***	520.08±45.99***abcde***
3w	124.77±14.56	161.67±17.87	258.28±13.61***a***	158.84±14.67	119.90±12.17***c***	252.18±48.78***ae***
CRP (µg/mL)						
0w	538.49±39.25	552.72±37.64	447.52±20.33	555.59±30.32	556.80±46.46	583.59±52.07
1w	687.91±40.71	592.39±52.52	859.51±35.17***ab***	705.95±49.94	637.29±30.70***c***	691.28±50.34***c***
3w	575.03±33.24	820.61±48.02***a***	767.56±27.10***a***	771.54±64.15***a***	605.91±42.61***b***	551.63±34.25***bcd***
ALT (U/L)						
0w	25.25±1.75	25.14±1.89	16.00±0.58	26.14±3.79	26.10±1.46	18.57±2.43
1w	34.50±1.78	33.14±1.91	33.67±1.99	27.00±4.99	31.00±1.17	36.00±2.73
3w	39.75±2.68	33.57±1.43	33.40±1.17	31.28±3.08	32.80±3.55	35.86±2.26
AST (U/L)						
0w	72.87±2.66	73.57±4.20	78.00±2.78	69.14±7.37	65.90±3.18	72.28±3.16
1w	84.00±3.09	88.43±6.13	79.17±1.85	71.00±9.41	72.10±4.26	78.14±3.13
3w	73.43±4.79	80.14±8.42	92.00±7.54	75.00±7.85	87.80±7.77	86.50±4.60

Results are presented as Mean ± SEM. a $p<0.05$ vs Sham group; b $p<0.05$ vs CRF group; c $p<0.05$ vs CRF+rHuEPO 100IU; d $p<0.05$ vs CRF+rHuEPO 200IU; e $p<0.05$ vs CRF+rHuEPO 400IU (Mann-Whitney test). 0w –start of protocol; 1w – 1 week after starting rHuEPO treatment; 3w – 3 weeks after starting rHuEPO treatment (end of protocol); ALT – alanine transaminase; AST - aspartate aminotransferase; CRP – C-reactive protein; Hb – hemoglobin; Ht – hematocrit; CRF – chronic renal failure; RBC – red blood cells; Ret – Reticulocytes.

CRF400 groups, in contrast with reduced expression in CRF100 and CRF600 groups (Fig. 4F). Concerning HIF system, we found an up-regulation of HIF-1α (Fig. 4G) and HIF-2α (Fig. 4H) in CRF and CRF600 groups, and an increased expression of EPO mRNA levels in the same groups (Fig. 4I); a down-regulation was observed for HIF-1α in CRF100, CRF200 and CRF400 groups (Fig. 4G), and the same was observed for EPO expression (Fig. 4I).

In the duodenum, a down-regulation of divalent metal iron transporter 1 (DMT1) mRNA levels (Fig. 4J) was found in all CRF groups, when compared with Sham group, except in CRF600 group, presenting an overexpression of DMT1 mRNA levels. The protein levels of FPN in the duodenum increased in a rHuEPO dose-dependent manner, except in the CRF600 group that presented a reduction, also found in the CRF group (Fig. 5B).

No differences were found in protein liver pERK1/2:ERK1/2 ratio between groups (Fig. 5C); however, an increase in protein liver pSMAD1/5/9: SMAD1/5/9 ratio (Fig. 5D) and a reduction in total protein SMAD4 (Fig. 5E) were found in the CRF100 and CRF600 groups, while CRF200 and CRF400 groups showed a significant increase.

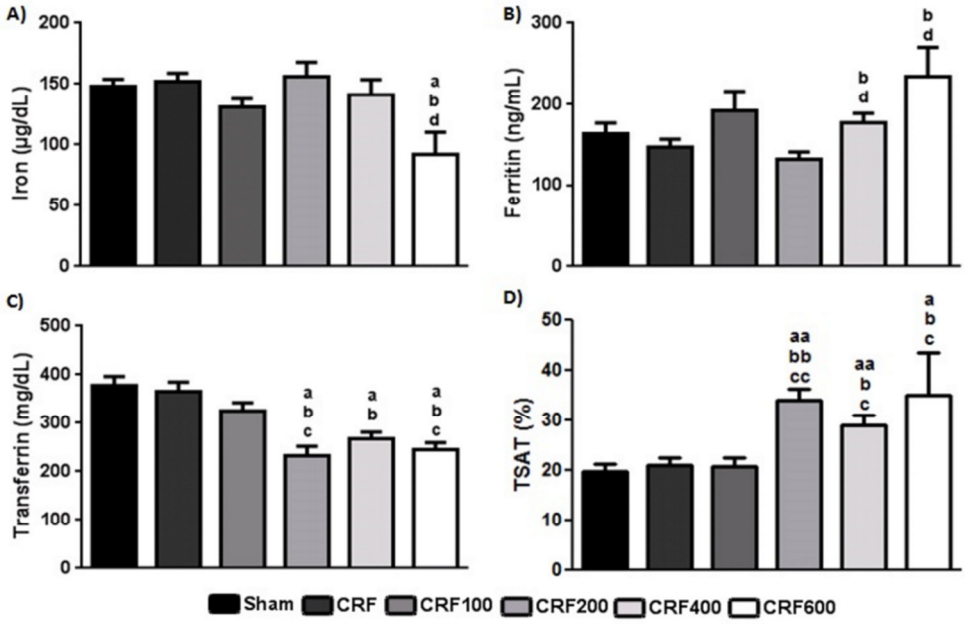

Fig. 2 – Iron data at the end of the protocol by group. Results are expressed as mean±SEM. a) $p<0.05$ vs Sham group, b) $p<0.05$ vs CRF group, c) $p<0.05$ vs CRF100 group, d) $p<0.05$ vs CRF200 group (Mann – Whitney test). CRF – chronic renal failure; TSAT – transferrin saturation.

4. Discussion

Under anemic conditions, the reduction of oxygen delivery to tissues leads to hypoxia, a recognized inhibitor of hepcidin transcription [4]. In our study, the CRF group developed anemia due to the reduced EPO renal production. No significant changes were observed, neither in serum iron, Tf and ferritin, nor in the gene expression of HJV and BMP6; in line with this, no significant changes were observed in SMAD1/5/9 phosphorylation and a significant increase was found for total SMAD4; however, a down-regulation in hepcidin gene expression was observed in the CRF group that seems to result from the up-regulation of HIF and (hepatic) EPO gene expressions, triggered to induce erythropoiesis. These findings are in accordance with recent reports by Mastrogiannaki et al. [23] and Ravasi, et al. [14]. Matriptase-2, known as negative modulator of hepcidin synthesis [24], was up-regulated under the hypoxic conditions in CRF rats, and may also contribute to reduce hepcidin mRNA levels. The reduction in hepcidin protein synthesis in the CRF group, though without statistical significance, was associated to a significant decrease in duodenal protein FPN and in the expression of DMT1 gene, in accordance with the normal values of serum iron, Tf and ferritin. The significant up-regulation in TfR1 and TfR2 is, thus, probably related to hypoxia [25].

The treatment with the lowest dose of rHuEPO (CRF100) triggered the increase in RBC production, as showed by the significantly higher values in all hematologic parameters under study, leading to normoxia. The erythropoietic rise did not significantly change serum iron, Tf and ferritin concentrations, explaining the significant down-regulation in Tfr1 and Tfr2 mRNA levels. Indeed, hepcidin protein synthesis and most of the factors regulating hepcidin reached to values similar to those presented by the Sham group, suggesting a main role for rHuEPO in the slight inhibition of hepcidin (Fig. 1).

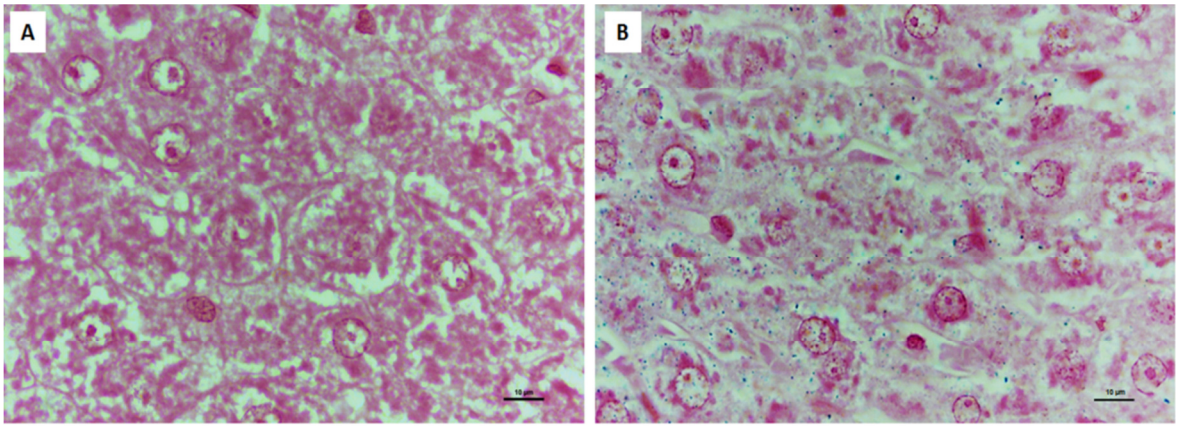

Fig. 3 – Liver sections of Sham (A) and CRF600 (B) groups demonstrating staining for hemosiderin (Perls Stain x100).

In the CRF200 group the production of reticulocytes was significantly increased in the first week of treatment with rHuEPO, and normal afterwards, with hematologic values similar to those observed in the Sham group; however, some changes were already observed in iron metabolism, namely, a significant reduction in hepcidin synthesis, leading to a significant increase in duodenal protein FPN, increasing iron absorption and, thus, increasing TSAT and inducing TfR1 and TfR2 mRNA levels. The higher levels of rHuEPO, the significant increase in matriptase-2 and the significant reduction in BMP6 gene expressions, associated with no increase in phosphorylated SMAD proteins, may explain the observed reduction in liver hepcidin protein.

In the CRF400 group, the increased rHuEPO dose triggered a stronger erythropoietic stimulus in the first week of treatment, leading to a significant increase in reticulocyte production. In spite of the continuous high exogenous erythropoietic stimulus by rHuEPO, after 3 weeks of treatment only slightly higher RBC values were observed. The reduction in liver hepcidin was still observed with this dose, as well as the changes in the others factors modulating its synthesis; the disturbances in iron metabolism, enhancing iron absorption and favouring the increase in iron stores were also observed.

With the highest rHuEPO dose (CRF600), an increase in reticulocyte number was observed after 1 week of treatment; however, the control on their production was weaker, as after 3 weeks of treatment the reticulocyte count and the RBC concentration was significantly higher than that observed in the Sham group. In spite of the up-regulation of HIF system, the high rHuEPO dose and the increasing erythropoiesis, known to inhibit hepcidin synthesis [26-28], we found a significant increase in liver hepcidin protein that seems to be triggered via BMP6;strengthening this hypothesis, we found a significant increase in SMAD1/5/9 phosphorylation and a slight reduction in total SMAD 4. This increase in hepcidin synthesis explains the reduction in duodenal protein FPN, triggering the reduction in iron absorption, as showed by the significant decrease in serum iron, the increase in ferritin and the increased hepatic iron stores, though no changes in liver function markers were found.

Iron liver can directly induce BMP6 expression and activate the BMP-SMAD signaling pathway [7], which is in agreement with our results (Fig 1). Moreover, the increased gene expression of TfR2 was not linked to an increase in HJV gene expression, and any changes in the ERK1/2 pathway were found supporting the hypothesis of a direct

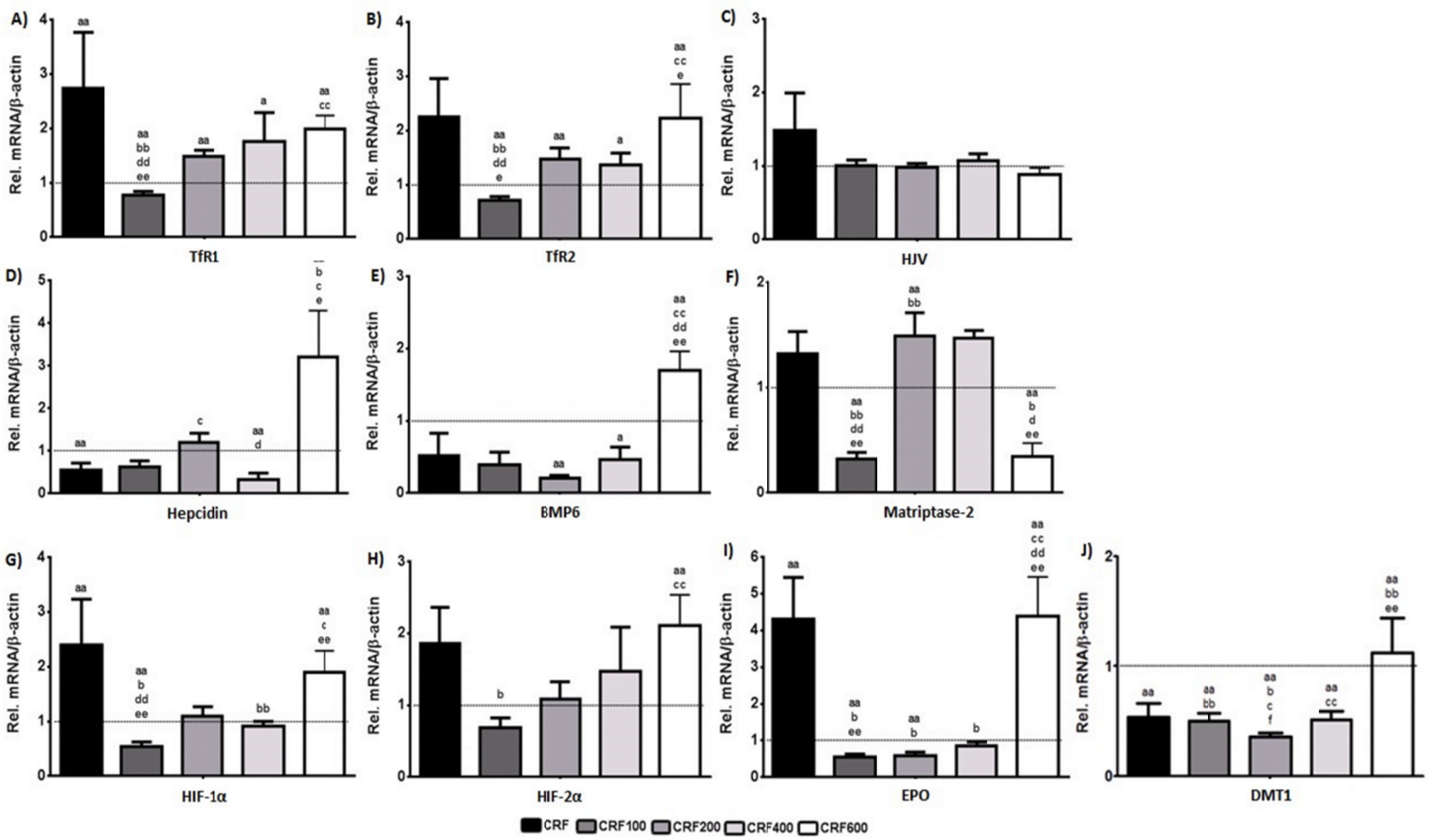

Fig. 4 – Relative mRNA expression of liver (A-I) and duodenum (J) genes involved in iron metabolism at the end of the protocol. Results are expressed as mean±SEM. a) $p<0.05$ vs Sham group, b) $p<0.05$ vs CRF group, c) $p<0.05$ vs CRF100 group, d) $p<0.05$ vs CRF200 group, e) $p<0.05$ vs CRF400 group, f) $p<0.05$ vs CRF600 (Mann-Whitney test). BMP6 – bone morphogenetic protein 6; DMT1 - divalent metal transporter 1; EPO – erythropoietin; Hamp – hepcidin; HIF1α – hypoxia induclible factor 1 alpha; HIF2α – hypoxia inducible factor 2 alpha; HJV – hemojuvelin; TfR – transferrin receptor; TMPRSS6 – matriptase-2.

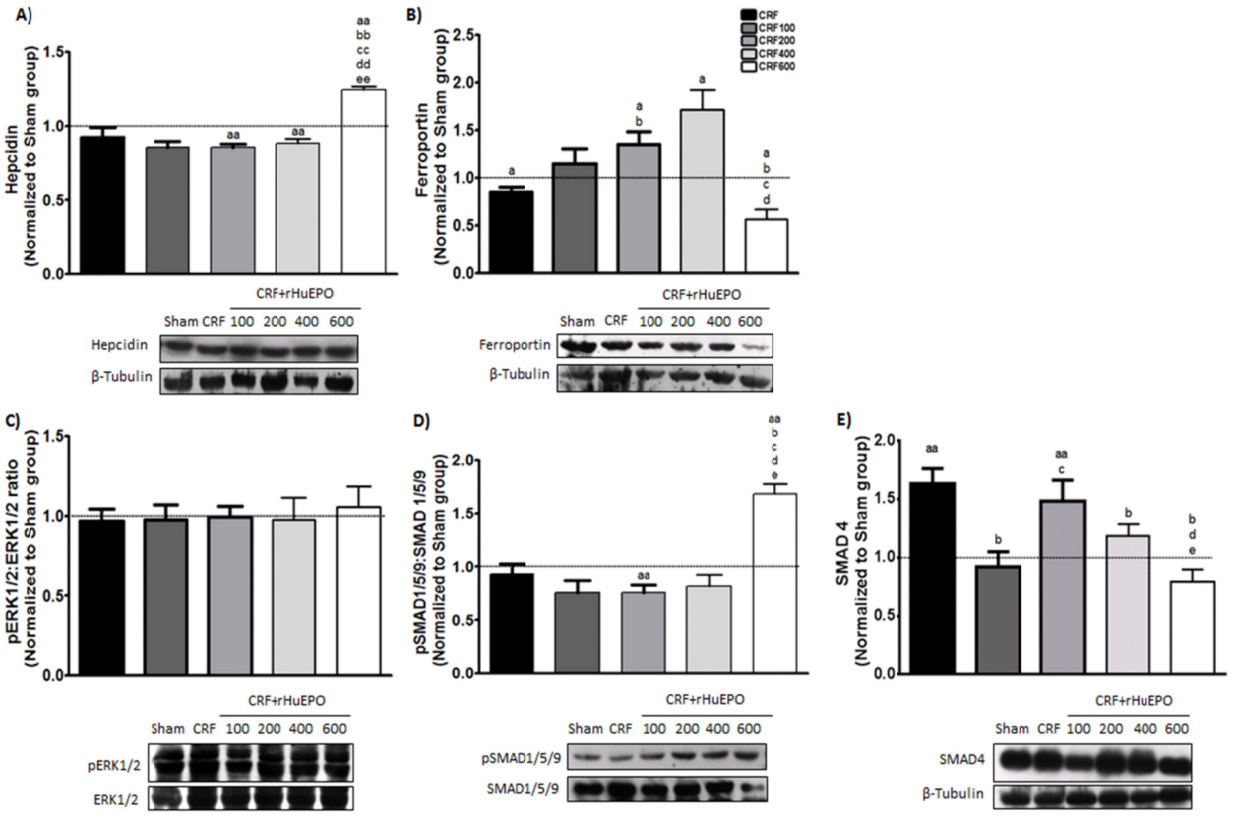

Fig. 5 – Evaluation of liver hepcidin protein (A), ferroportin in duodenum (B) and the signaling pathways of hepcidin in liver -pERK1/2:ERK1/2 ratio (C), pSMAD1/5/8:SMAD1/5/8 (D) and SMAD4 (E) – by western blotting. Results are expressed as mean±SEM. a) $p<0.05$ vs Sham group, b) $p<0.05$ vs CRF group, c) $p<0.05$ vs CRF100 group, d) $p<0.05$ vs CRF200 group, e) $p<0.05$ vs CRF400 group (Mann- Whitney test). CRF – chronic renal failure; rHuEPO – recombinant human erythropoietin.

action of iron liver (and not of TSAT) in the regulation of hepcidin synthesis. Diaz et al. reported similar findings, using a model of accelerated erythropoiesis [29]. Increased levels of BMP2 have been also reported [30], though BMP6 seems to be the most important BMP in hepcidin regulation [31].

Our data suggest that the rHuEPO stimuli trigger a first wave to achieve correction of the anemia/hypoxia, by inhibiting hepcidin synthesis, favouring erythropoiesis through an increase in iron absorption. In spite of the continuous exogenous increasing rHuEPO stimulus, the bone marrow stroma is able to control erythropoiesis; however, the iron stores will continuously increase, due to the increased iron absorption. When the ability of the bone marrow stroma fails to control abnormally high erythropoietic stimulus (CRF600), erythrocytosis develops and the increased hepatic iron stores are able to trigger hepcidin synthesis via BMP6. Thus, hepatic iron-induced hepcidin synthesis prevails over the other factors regulating hepcidin synthesis (Fig. 1).

In our model, the increase in hepcidin due to an inflammatory condition should be excluded, as the CRF600 group presented CRP levels similar to Sham group. As reported by others, in a moderate degree of renal insufficiency hepcidin has been correlated with iron stores, but not with inflammatory markers [32]. In addition, the increase in hepcidin is not due to reduced excretion, as no difference in glomerular filtration rate between all CRF groups was found (data not shown).

Recent studies raised concerns about the increased proportion of patients with serum ferritin values ≥800 ng/mL [33], and tissue iron accumulation (liver and spleen) in CKD patients [34] that may lead to further and more severe iron disturbances. Iron stores are usually evaluated by measuring ferritin blood levels; however, ferritin and liver iron are not always correlated, as some patients with ferritin levels below 500 ng/mL may present liver iron accumulation [35]. In a study by our group, using a rat model of nephrectomy, we found iron deposition in renal tubules, along with extensive tubulointerstitial lesions that could be responsible for CKD progression [22].

Our work raises the hypothesis that when high inflammation (triggering hepcidin synthesis) is associated with increased iron stores in HD patients, hepcidin synthesis is enhanced through BMP6, leading, therefore, to worsening of anemia and, eventually, to a hyporesponsiveness to ESA therapy.

In summary, our data suggest that liver iron overload is an important stimulus for hepcidin synthesis, stronger than the inhibitory effect of high rHuEPO doses (Fig.1), and that liver iron overload should be further studied as a potential factor for the development of a hyporesponse to rHuEPO therapy. Moreover, the search for new, and more sensitive, biomarkers of tissue iron overload is warranted.

Conflict of interest

No relevant conflicts of interest to disclose.

Acknowledgements

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Paper III

Pathological and molecular mechanisms underlying resistance to recombinant human erythropoietin therapy in the remnant kidney rat model of chronic kidney disease associated anemia

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Pathological and molecular mechanisms underlying resistance to recombinant human erythropoietin therapy in the remnant kidney rat model of chronic kidney disease associated anemia

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ABSTRACT

Anemia of chronic kidney disease (CKD) can be corrected by treatment with recombinant human erythropoietin (rHuEPO); however, some patients become hyporesponsive. The molecular mechanisms underlying this resistance remain to be elucidated. Our aim was to study hyporesponsiveness to rHuEPO therapy using the remnant kidney rat model of anemia associated with CKD induced by 5/6 nephrectomy.

At starting, male Wistar rats were divided in 3 groups, for a 3-week protocol: Sham, CRF (vehicle) and rHuEPO (200IU/kg body weight [BW]/week) treated groups; at the end of protocol, the rHuEPO treated rats were subdivided in responders (CRF200) and non-responders (CRF200NR), according to their hematologic response; blood, cellular and tissue studies were performed. The CRF200 group achieved correction of anemia, while the CRF200NR group developed anemia, after an initial response (1st week) to rHuEPO therapy. CRF and CRF200NR groups presented the highest serum CRP levels; CRF200NR showed also high levels of renal inflammatory markers, such as interleukin (IL)-6, IL-1β, nuclear factor kappa B, connective tissue growth factor (CTGF) and transforming growth factor beta 1 (TGF-β1); no changes were found in iron metabolism. Our data suggest that the development of anemia/rHuEPO hyporesponsiveness is associated with a higher systemic and renal inflammatory condition, favoring hypoxia and triggering an increase in renal expression of HIF-1α, TGF-β1 and CTGF that will further aggravate renal fibrosis, which will further enhance the inflammatory response, creating a cycle that promotes disease progression. New therapeutic strategies to reduce inflammation in CKD patients could improve the response to rHuEPO therapy and reduce hyporesponsiveness.

Keywords: anemia; CTGF; erythropoietin; hyporesponsiveness; inflammation; TGF-β1

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1. Introduction

Anemia is a common complication in chronic kidney disease (CKD) patients that develops early in the course of the disease, increasing its frequency and severity with the progression of CKD [1]. Worsening of anemia has been associated with increasing morbidity and mortality, mainly, in patients under dialysis therapy [2-4]. Several factors contribute to CKD anemia, including inflammation, reduced red blood cell (RBC) life span, iron deficiency, uremic toxins, as well as, erythropoietin (EPO) deficiency, which is the major cause [5]. In the adult life, the kidney is the major organ producing EPO and the liver a secondary one [6]. The production of EPO is regulated by hypoxia, through the hypoxia inducible-factor (HIF) system, including the oxygen-dependent regulated subunits (HIF-1α, HIF-2α and HIF-3α) and the constitutive subunits (HIF-1β and HIF-2β) [7]. Under normoxia, HIF-2α is hydroxylated by an oxygen dependent prolyl-hydroxilase and, afterwards, the von Hippel–Lindau tumor suppressor protein targets the HIF-α subunit for rapid ubiquitination and proteasomal degradation. However, under hypoxic conditions the HIF-α subunits that are not hydroxylated, accumulate and translocate to the nucleus forming a complex with HIF-β activating the transcription of EPO gene and of other HIF target genes [7]. HIF-1α and HIF-2α are similar, but their target genes are different, HIF-1α regulates glycolytic genes and pro-fibrotic genes, whereas HIF-2α is the main responsible for EPO production [7]. An acute activation of the HIF system by tissue hypoxia leads to a protective response; however, a continuous activation, as occurs in CKD, can contribute to the progression of the disease, through the enhancement of renal fibrosis [8], which is the common final feature in CKD disease, irrespective of the initial cause [9].

According to the clinical practice guidelines, anemia of CKD patients can be corrected by the use of erythropoiesis-stimulating agents (ESA), such as recombinant human erythropoietin (rHuEPO) [10]. EPO and ESA stimulate erythropoiesis through the activation of a homodimer EPO receptor (EPOR); several pleiotropic effects, such as renoprotection, are also attributed to ESA treatment, due to their interaction with an heterodimer EPOR [11]. The majority of CKD patients respond to ESA therapy; however, about 5–10% of CKD patients are hyporesponsive [12], which has been associated with increased risk of cardiovascular events and mortality [13-15]. According to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines [10], CKD patients can present initial or acquired ESA hyporesponsiveness. In a primary hyporesponse to ESA treatment, the patient presents no increase in hemoglobin (Hb) concentration after one month of treatment with adequate weight-based ESA dose; an acquired ESA resistance develops when, after treatment with a stable ESA dose achieving target Hb concentration, the patient requires two increases in ESA dose (up to 50% beyond the stable dose) to achieve Hb concentration. Several factors can contribute to this condition, such as iron deficiency (absolute or functional), inflammation and uremic toxins, among others [16,17]. Inflammation is a common feature in CKD patients, mainly in those on dialysis therapy [15,17] and has been associated with increased levels of hepcidin [18]; this liver peptide regulates iron metabolism by degrading ferroportin (an iron exporter), present in the membrane surface of enterocytes, hepatocytes and macrophages; thus, hepcidin controls iron absorption and mobilization from iron stores [19,20]. Increased hepcidin levels lead to a functional iron deficiency, due to a reduced iron absorption and mobilization and to an increase in ferritin levels [21]. The molecular mechanisms linking these factors that may underlie the development of a hyporesponse to ESA remain to be elucidated. This is, probably, due to the

lack of well-designed in vivo studies that cannot be performed in humans, due to ethical reasons. In that way, we used the 5/6 nephrectomy-induced remnant kidney rat model of CKD-associated anemia under rHuEPO therapy, to study the underlying causes of hyporesponsiveness to ESA therapy, focusing on iron metabolism, renal damage, hypoxia, inflammation and fibrosis.

2. Animals and methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab., Inc., Chatillon-sur-Chalaronne, France), 12 weeks old, were maintained in ventilated cages, in an air conditioned room, subjected to 12h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. The rats were randomly initially divided in three groups: a sham operated group (n=8), subjected to surgical process but without kidney mass reduction, and two chronic renal failure (CRF) groups induced by a two-stage (5/6) nephrectomy, with surgical excision of both poles of the left kidney (2/3 nephrectomy) by left flank incision and, one week later, complete removal of the right kidney through identical procedure. After another week, while one of the CRF groups remained under vehicle treatment (saline solution – CRF group, n=7), the other group started subcutaneous (sc) rHuEPO (200 IU/Kg body weight [BW]/week of NeoRecormon®, Roche Pharmaceuticals, Basel, Switzerland) treatment, 3 times per week, for further 3 weeks. At the end of protocol and according to the hematological response, the rHuEPO-treated group was further subdivided in responder (CRF200, n=7) or non-responder (CRF200NR, n=5). To define non-response to rHuEPO we used, as the cut-off value, the highest value of Hb concentration observed in the CRF group. Thus, the rats presenting an Hb concentration lower than the cut-off value, were classified as non-responders. All animals received human care and animal experiments were conducted according to the European Communities Council Directives on Animal Care. The experiments were approved by the Portuguese Foundation for Science and Technology and the Local Ethics Committee (ORBEA: Organ Responsible for Animal Welfare) of the Faculty of Medicine from the University of Coimbra.

2.2. Sample collection

Blood samples were collected at baseline, one week after starting treatment and at the end of the protocol (3 weeks of treatment) with rats under anesthesia (intraperitoneal) with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncuture, from the jugular vein, into Vacuette® (Greiner Bio-One GmbH, Germany) tubes without anticoagulant (to obtain serum) or with K_3EDTA for hematological and biochemical studies. Aliquots of serum and plasma were immediately stored at -80ºC until assayed.

At the end of protocol, after blood collection, and under anesthesia, rats were sacrificed by cervical dislocation, and the kidney, the liver and the duodenum were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighted. A bone marrow aspirate from the femur was also performed. In order to isolate total RNA, small portions of kidney, liver and duodenum from each rat, were immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) upon collection and stored at 4°C for 24h; afterwards, samples were frozen at -20ºC. For western blot analysis organs were immediately frozen with liquid nitrogen and stored at -80ºC.

2.3. Biochemical and hematological assays

RBC count, Hb, hematocrit (Ht), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and platelet count (PLT) were assessed in whole blood K_3EDTA, using an automated blood cell counter (HORIBA ABX, Amadora, Portugal). Reticulocyte count was determined by microscopic counting on blood smears after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA).

Reticulocyte production index (RPI) was calculated as previously described by Hillman and Finch [22] [(reticulocyte %/maturation index)* (Ht/normal Ht)], where normal Ht was the mean value presented by the Sham group, and the maturation index (maturation time of circulating blood reticulocytes that increases with increasing production and/or with premature release of reticulocytes from the bone marrow) was 1 for Sham and 1.5 for anemic groups.

Serum EPO and C-reactive protein (CRP) levels were evaluated by rat specific ELISA kits, according to manufacture instructions (MyBioSource, San Diego, CA, USA and eBioscince, San Diego, CA, USA, respectively).

Serum blood urea nitrogen (BUN), creatinine and uric acid were analyzed through automatic methods and equipments (Hitachi 717 Chemistry Analyzer, Roche Diagnostics, Basel, Switzerland).

Serum iron and ferritin were analyzed through automatic methods and equipments (Roche Integra 400, Roche Diagnostics, Basel, Switzerland). Serum transferrin (Tf) levels were evaluated by rat specific ELISA kit (Transferrin Rat ELISA Kit, abcam, Cambridge, UK). Tf saturation (TSAT) was calculated using the formula (Iron µg/dL*100)/(Tf mg/dL*2).

2.4. Gene expression analysis

Kidney, liver and duodenum RNA isolation and integrity control were performed as previously described [23]. One µg of total RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer instructions. One ng of cDNA was used for gene expression analysis with qPCR using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs (Table 1).

Table 1 - List of primer sequences and annealing temperatures

Gene	Primer sequences (5' → 3')	Annealing temperature
actb	F: TACAGCTTCACCACCACA GC R: AAGGAAGGCTGG AAGAGA GC	57ºC
bmp6	F: GGTGGAGTACGACAAGGA GTT R: GTCACAACCCACAGATTGCTA	56ºC
ctgf	F: CGTAGACGGTAAAGCAATGG R: AGTCAAAGAAGCAGCAAACAC	60ºC
epas1	F: TGACTTCACTCATCCTTGCGACCA R: ATTCATAGGCAGAGCGGCCAAGTA	59ºC
gapdh	F: TGCCACTCAGAAGACTGTGG R: ACGGATACATTGGGGGTAGG	59ºC
hamp	F: GGCAGAAAGCAAGACTGATGAC R: ACAGGAATAAATAATGGGGCG	58ºC
hfe2	F: TTCCAATCCTGCCTCTTTGAT R: GGAAAAGGTGCAAGTTCTCCAA	58ºC
il6	F: ATGTTGTTGACAGCCACTGC R: TTTTCTGACAGTGCATCATCG	58ºC
slc11a2	F: ATAGCAGACGCCCCCATG R: AGGCCCGAAGTAACATCCAA	58ºC
tfrc	F: GGGAGCCATTGTCATACACC R: GTCGCAAAGCAGAGTCTTCC	58ºC
tfr2	F: AGCTGGGACGGAGGTGACTT R: TCCAGGCTCACGTACACAACAG	58ºC
tmprss6	F: AGAAGGTGGATGTGCAACTGATC R: CTTGCCCTTGCGATAACCA	59ºC

F: Forward; R: Reverse; *actb* - Beta-actin; *bmp6* - bone morphogenetic protein 6; *ctgf* - connective tissue growth factor; *epas1*- endothelial PAS domain protein 1; *gapdh* - glyceraldehyde 3-phosphate dehydrogenase*; hamp* – hepcidin; *hfe2* - hemojuvelin; *il6* – interleukin 6; *slc11a2* - divalent metal transporter 1*; tfrc* – transferrin receptor 1; *tfr2* – transferrin receptor 2; *tmprss6* – matriptase-2.

qPCR reactions were performed using the following conditions: enzyme activation at 95ºC for 3min; denaturation at 95ºC for 3sec; annealing for 30sec (for each pair primer temperature refer to Table 1).

Gene expression was normalized to a*ctb* (beta actin) and *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) genes, and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. In the liver, the expression of the following genes was analyzed: *bmp6* (bone morphogenetic protein 6), *epas1* (endothelial PAS domain-containing protein 1, HIF2α), *hamp* (hepcidin), *hfe2* (hemojuvelin), *tfrc* (TF receptor (TfR) 1), *tfr2* (TfR2) and *tmprss6* (matriptase-2). In the kidney the expression of *ctgf* (connective tissue growth factor) and *il6* (interleukin (IL)-6) genes was analyzed. In the duodenum, the expression of the *slc11a2* (divalent metal transporter 1) gene was evaluated.

2.5. Western blot analysis

Liver and kidney proteins were extracted using RIPA buffer (NaCl 150mM, Tris-HCl 50mM pH8, Triton X-100 1%, ethylene glycol tetraacetic acid 5mM, deoxycholic acid 0.5%, sodium lauryl sulfate 0.1%) and ultra-sonication. After centrifugation, the protein concentration of the supernatant was assayed using the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, IL, USA). Aliquots of the extract containing 50-100 µg of proteins were separated by reducing SDS-PAGE (10%) and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked by using 7% non-fat milk in a solution of Tris-buffered salt with Tween-20. The membranes were incubated, overnight at 4ºC, with rabbit polyclonal antibody anti-EPO 1:200 (sc-7956), anti-EPOR 1:200 (sc-697, Santa Cruz Biotechnoloy, Inc, TX, USA) and with goat polyclonal antibody anti-IL1β 1:1000 (AF-501-NA, R&D Systems, MN, USA); afterwards, they were incubated with anti-goat (ab97120) or anti-rabbit (sc-2004) secondary antibody-conjugated with horseradish peroxide 1:1000 (abcam, Cambridge, UK and Santa Cruz Biotechnoloy, Inc, TX, USA), respectively. Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL; WesternBright, Advansta, CA, USA). The immunoblots analysis was performed by densitometry (Bio1D++ version 99, Vilber Lourmat). To ensure even loading of the samples, membranes were probed with rabbit anti-β-tubulin antibody 1:500 (sc-9104, SantaCruz Biotechnology, TX, USA). The protein concentration found for each sample was normalized for the protein concentration observed in the Sham group.

2.6. Histopathological analysis

Kidney samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3µm thick sections were stained with Periodic acid of Shiff (PAS). All samples were examined by light microscopy using a Microscope Zeiss Axioplan 2 and images were captured using a digital microscope camera (Leica DFC450). Lesions were evaluated in a double-blinded fashion by the pathologist, who evaluated and quantified lesions as previously described [23].

2.7. Immunohistochemistry

Tissue (kidney and liver) sections were incubated in xylene, rehydrated via graded ethanol series to water. To retrieve antigen exposure, the samples were treated with 0.01M citrate buffer solution at 95°C to during 45min. The samples were processed for indirect immune detection using a mouse/rabbit specific horseradish peroxidase (HRP)/ diaminobenzidine (DAB) detection immunohistochemistry (IHC) kit (ab80436, Abcam Inc, Cambridge, UK), according to the manufactor's protocol. Negative controls were included in each experiment, by omission of the primary antibodies. Polyclonal rabbit

antibody anti-HIF-1α 1:200 (sc-53546), anti-EPAS-1 (HIF-2α) 1:500 (sc-28706), anti-EPO 1:200 (sc-7956), anti-nuclear factor kappa B (NF-κB) p65 1:100 (sc-109) and anti-transforming growth factor beta 1 (TGF-β1) 1:100 (sc-146) diluted in TBS-Tween 0.05% served as primary antibodies and were incubated overnight at 4°C in a humidified chamber. Excessive, unbound primary antibody was removed by washing with TBS-Tween 0.05% buffer. Sections were counterstained with hematoxylin, dehydrated, and mounted in a non-aqueous media (DPX Mountant for Microscopy, VWR BDH Prolabo, PA, USA) and examined with a microscope Nikon Eclipse. Images were captured using a digital microscope camera (Nikon DS-Ri2). Immunopositivity was scored in accordance to brown staining intensity (I) and percentage of positive cells or area (P), as previously described [23]. *2.8. Statistical analysis* *R*esults are presented as mean ± standard error mean (SEM). For comparison between groups Mann-Whitney U test was performed. Statistical significance was accepted at $p<0.05$. Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS) for Windows, version 22.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Hematological and serum biochemical data

After one week of nephrectomy the CRF group presented a reduction in RBC count, Hb concentration and Ht, when compared to Sham group, reflecting the development of anemia that remained until the end of protocol (Table 2). MCHC showed an increase at both follow-up times, compared to Sham rats. No significant alterations were found in the other hematological parameters (Table 2).

The introduction of rHuEPO treatment corrected anemia after one week in the CRF200 group, as showed by the increase in RBC count, Hb concentration and Ht, which were further increased at the end of protocol, to values that were similar to those presented by Sham group. The CRF200NR group showed a slight correction of the anemic state after one week of treatment; however, at the end of the experimental protocol, RBC count, Hb concentration and Ht showed a reduction to values that were similar to those found in the CRF group. Reticulocytes and RPI values in the CRF group remained stable and similar to those observed for the Sham group along the study. In the CRF200 and CRF200NR groups, after one week of rHuEPO treatment reticulocytes and RPI values increased significantly, but at the end of protocol, their values were similar to those of Sham and CRF groups, except the RPI value in the CRF200NR group, which was significantly lower versus Sham and CRF200 groups (Table 2). Both rHuEPO-treated groups presented reduced MCV values and increased MCHC levels at the end of protocol; the CRF group presented also a trend towards an increase in MCV and significantly higher values of MCHC.

Regarding serum EPO levels, we found that the CRF group presented significantly increased values along the study (more than 2 fold the Sham value, at the end of protocol); the CRF200NR also showed increased serum EPO levels after 1 week of treatment that reached statistical significance at 3 weeks of treatment; the CRF200 presented a significant increase at the 1st week and decreased afterwards (Table 2).

The microscopic analysis of bone marrow aspirates examination of rats showed that in CRF200NR the proportion of red-cell precursors was significantly lower than in Sham and in the other CRF groups. The erythroid:myeloid (E:M) ratio was 1:2 for the Sham group and CRF group, while for the CRF200NR group the ratio was 1:7 the CRF200 presented a E:M ratio of 2:1.

Serum urea and creatinine concentrations were significantly higher in all CRF groups, at 1 and 3

Table 2 – Hematological data throughout the study (at baseline, o weeks, and after 1 and 3 weeks of treatment)

		Sham (n=8)	CRF (n=7)	CRF200R (n=7)	CRF200NR (n=5)
		Hematological data			
RBC (x 10^9/L)					
	0w	7.41±0.07	7.43±0.09	7.53±0.11	7.66±0.13
	1w	7.11±0.08	6.11±0.12***a***	6.80±0.14***b***	6.68±0.07***ab***
	3w	7.77±0.13	6.41±0.11***a***	7.48±0.17***b***	6.22±0.43**ac**
Hb (g/dL)					
	0w	13.85±0.13	13.63±0.18	13.54±0.17	13.62±0.19
	1w	13.94±0.11	12.26±0.22***a***	13.86±0.20***b***	13.58±0.29***b***
	3w	14.07±0.15	12.06±0.18***a***	13.89±0.32***b***	11.60±0.75***ac***
Ht (%)					
	0w	41.12±0.54	41.34±0.59	40.90±0.55	41.32±0.66
	1w	38.02±0.20	32.13±0.63***a***	36.21±0.92***b***	35.54±0.82***ab***
	3w	41.77±0.80	33.56±0.57***a***	38.03±0.87***b***	30.90±2.38***ac***
Ret (%)					
	0w	1.95±0.17	2.47±0.29	2.34±0.23	3.02±0.60
	1w	1.73±0.31	2.18±0.23	5.27±0.35***ab***	5.28±0.71***ab***
	3w	1.61±0.19	2.53±0.28***a***	2.14±0.21	1.84±0.33
Ret (x10^9/L)					
	0w	144.39±12.38	183.41±20.89	177.06±18.25	233.48±49.15
	1w	122.47±22.47	134.11±15.26	358.17±25.05***ab***	352.66±46.76***b***
	3w	124.77±14.56	161.67±17.87	158.84±14.67	112.82±17.66
RPI					
	0w	1.94±0.16	2.47±0.28	2.34±0.23	3.04±0.61
	1w	1.72±0.31	1.34±0.23	4.86±0.51***ab***	4.50±0.46***ab***
	3w	1.60±0.19	1.37±0.15	1.93±0.17	0.90±0.14***ac***
MCV (fL)					
	0w	55.75±0.53	55.57±0.30	54.29±0.36	54.00±0.71
	1w	53.62±0.59	52.57±0.65	53.28±0.47	53.20±0.97
	3w	53.50±0.42	52.28±0.28	51.00±0.31***ab***	50.00±0.95a***b***
MCH (pg)					
	0w	18.69±0.19	18.34±0.24	18.03±0.26	17.74±0.31
	1w	19.66±0.34	20.11±0.38	20.44±0.27	20.38±0.40
	3w	18.16±0.32	18.80±0.23	18.58±0.33	18.74±0.25
MCHC (g/dL)					
	0w	33.65±0.16	32.97±0.41	33.13±0.25	32.92±0.43
	1w	36.62±0.26	38.20±0.36***a***	38.37±0.46***a***	38.30±0.31***a***
	3w	33.80±0.71	35.94±0.30***a***	36.47±0.54***a***	37.68±0.53***ab***
Serum EPO (mIU/mL)					
	0w	2.48±0.09	3.04±0.22	3.23±0.25	3.35±0.72
	1w	3.52±0.39	5.68±0.41***a***	5.44±0.46***a***	4.71±0.48
	3w	4.38±0.53	9.71±1.28***ac***	4.55±0.26	6.35±0.51***ac***

Results are presented as mean ± SEM. a p<0.05 vs Sham group; b p<0.05 vs CRF group; c p<0.05 vs CRF200 group; d p<0.05 vs CRF200NR group (Mann-Whitney test). 0w –start of protocol; 1w – 1 week after the start of rHuEPO treatment; 3w – 3 weeks after the start of rHuEPO treatment (end of protocol); CRF – chronic renal failure; EPO – erythropoietin; Hb – hemoglobin; Ht – hematocrit; MCH – mean cell hemoglobin; MCHC – mean cell hemoglobin concentration; MCV – mean cell volume; RBC – red blood cells; Ret – reticulocytes; RPI – reticulocyte production index.

weeks of protocol, when compared to the Sham group (Table 3). No difference in serum urea was found between CRF groups; however, serum creatinine was significantly increased in the CRF200NR, when compared to the CRF and the CRF200 groups. No significant changes were found in uric acid values. At the end of protocol, all the CRF groups presented increased serum CRP levels, compared to the Sham group; the CRF and CRF200NR groups showed the highest PCR values (Table 3).

3.2. Body and tissue weights

All CRF groups showed significantly reduced BW at the end of the study, when compared to Sham

group (CRF: 0.389±0.007 Kg, CRF200: 0.369±0.010 Kg, CRF200NR: 0.343±0.019 Kg *versus* Sham: 0.420±0.07 Kg, $p<0.05$). Moreover, all CRF groups presented an increased kidney weight (KW)/BW ratio, when compared to Sham group (CRF: 3.827±0.185 g/Kg, CRF200: 3.441±0.167 g/Kg, CRF200NR: 4.751±0.996 g/Kg versus Sham: 2.767±0.071 g/Kg, $p<0.05$).

3.3. Iron metabolism

No significant alterations were found in serum iron and ferritin levels between groups (Fig. 1A1 and 1A2). Tf concentration (Fig. 1A3) was significantly reduced and TSAT (Fig. 1A4) was significantly increased in both CRF groups under rHuEPO treatment, when compared to Sham and CRF groups.

Liver TfR1 (Fig. 1B1) was significantly up-regulated in the CRF and CRF200 groups, when compared to Sham group; the CRF200NR group presented a down-regulation in TfR1, as compared to CRF and CRF200 groups. Liver TfR2 (Fig. 1B2) mRNA levels were increased in the CRF and in both CRF200 groups, reaching statistical significance only for CRF200 group. A significant down-regulation of HJV (Fig. 1B3) was observed in the CRF200NR, when compared to the other groups. A significant down-regulation of BMP6 (Fig. 1B4) (Fig. 1B5) was observed for all the CRF groups; a significant up-regulation of matriptase-2 was found in CRF200 rats and a significant down-regulation of hecpidin (Hamp) mRNA levels was observed in the CRF and CFR200NR animals (Fig. 1B6). Liver HIF-2α mRNA levels (Fig. 1B7) were up-regulated in the CRF and CRF200NR group. Regarding duodenal DMT1 mRNA levels (Fig. 1B8), a significant down-regulation was observed for the CRF and CRF200 groups, whereas in the CRF200NR a trend for increased mRNA levels was found that was significantly higher versus the CRF200 group.

Table 3 – Serum biochemical data throughout the study (at baseline, o weeks, and after 1 and 3 weeks of treatment)

	Sham (n=8)	**CRF (n=7)**	**CRF200 (n=7)**	**CRF200NR (n=5)**
Urea (mg/dL)				
0w	22.54±0.83	22.71±0.87	24.07±0.77	22.16±1.00
1w	23.07±0.64	56.33±2.40***a***	56.78±2.41***a***	53.23±7.52***a***
3w	22.64±0.91	54.10±3.63***a***	56.63±1.97***a***	54.67±5.76***a***
Creatinine (mg/dL)				
0w	0.37±0.01	0.35±0.01	0.39±0.01	0.38±0.02
1w	0.33±0.01	0.99±0.08***a***	1.34±0.16***a***	1.94±0.40***ab***
3w	0.45±0.04	1.00±0.07***a***	1.27±0.23***a***	2.21±0.62***abc***
Uric acid (mg/dL)				
0w	0.82±0.06	0.84±0.08	0.86±0.08	0.72±0.06
1w	0.79±0.07	0.91±0.09	0.87±0.04	0.76±0.07
3w	0.78±0.10	1.08±0.12	1.28±0.23	0.83±0.06
CRP (µg/mL)				
0w	538.5±39.3	552.7±37.6	555.6±30.3	542.1±27.7
1w	687.9±40.7	592.4±52.5	706.0±49.9	703.5±28.5
3w	575.0±33.2	820.6±48.0***a***	771.5±64.2***a***	803.0±39.8***a***

Results are presented as mean ± SEM. a $p<0.05$ vs Sham group; b $p<0.05$ vs CRF group; c $p<0.05$ vs CRF200 group; d $p<0.05$ vs CRF200NR group (Mann-Whitney test). 0w –start of protocol; 1w – 1 week after the start of rHuEPO treatment; 3w – 3 weeks after the start of rHuEPO treatment (end of protocol); CRF – chronic renal failure; CRP – C-reactive protein.

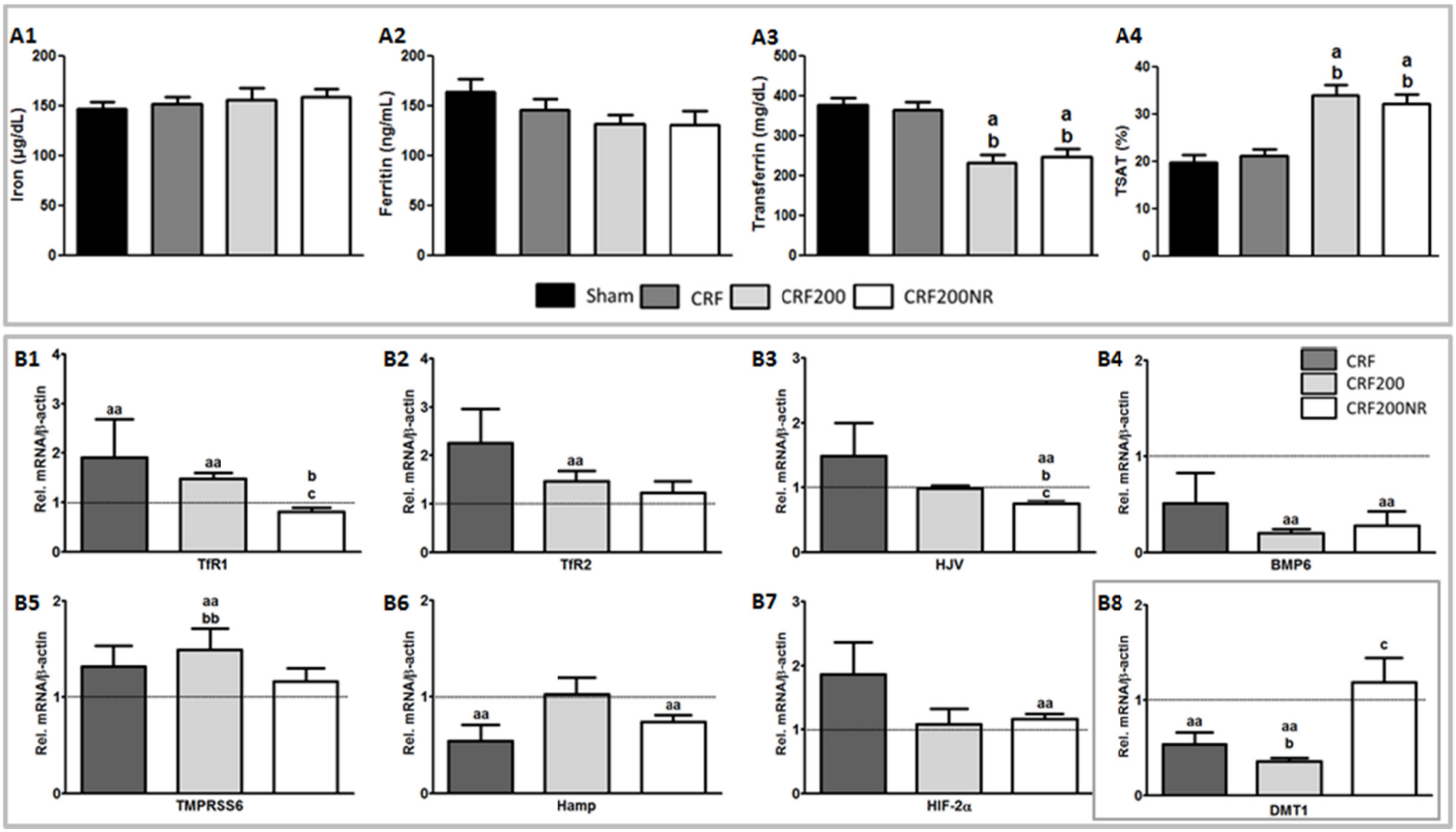

Fig. 1 – Iron metabolism (A1-A4) and relative mRNA expression of liver (B1-B7) and duodenum (B8) genes involved in iron metabolism, at the end of the protocol. Results are presented as mean ± SEM. a $p<0.05$, aa $p<0.01$ vs Sham group, b $p<0.05$, bb $p<0.01$ vs CRF group, c $p<0.05$ vs CRF200 group (Mann-Whitney test). BMP6 – Bone morphogenetic protein 6; DMT1 - divalent metal transporter 1; Hamp – hepcidin; HIF-2α – hypoxia inducible factor 2 alpha; HJV – hemojuvelin; TfR1 – tranferrin receptor 1; TfR2 – tranferrin receptor 2; TMPRSS6 – matriptase-2; TSAT – transferrin saturation.

3.4. Histopathological findings

The histopathological analysis of the kidney showed that the CRF group presented the highest total score for glomerular (Fig. 2A1 and 2A2), tubulointerstitial (Fig. 2A3 and 2A4) and vascular lesions (Fig. 2A5). All animals in this group presented glomerulosclerosis and tubular atrophy, accompanied by the presence of hyaline and granular cylinders, tubular dilations, calcification and necrosis, as well as, interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammatory infiltrate (Table 4). The presence of tubular necrosis hampers the observation of hydropic and vacuolar tubular degeneration, as these lesions precede tubular necrosis. Arteriolosclerosis and arteriosclerosis were also found in all rats from CRF group (Table 4). Both CRF groups under rHuEPO treatment presented an improvement in all the lesions, when compared to the CRF group, as showed by the reduced total scores (Fig. 2A1 – 2A5); however, a significantly higher score for mild glomerular lesions was observed in the CRF200NR group, when compared to the CRF200 group (Fig. 2A2) that results from the increased score for glomerular atrophy and dilation of Bowman´s space (Table 4). The histological data was similar for the two rHuEPO-treated groups, excepting for the tubulointerstitial lesions that presented a higher score for hyaline cylinders and IFTA in the CRF200NR group (Table 4). Tubular dilations and interstitial inflammatory infiltrate were observed in both groups with a similar total score (Table 4). Arteriolosclerosis showed also a trend towards higher scores in CRF200NR group.

Table 4 – Scoring of glomerular, tubulointerstitial and vascular kidneys lesions by group at the end of protocol

	Sham (n=8)	**CRF (n=7)**	**CRF200 (n=7)**	**CRF200NR (n=5)**
Advanced glomerular lesions				
Thickening of GBM	0	0.00±0.00	0.00±0.00	0.00±0.00
Mesangial expansion	0	0.00±0.00	0.00±0.00	0.40±0.40
Nodular sclerosis	0	0.00±0.00	0.00±0.00	0.00±0.00
Global Glomerulosclerosis	0	3.00±0.00***a***	0.43±0.43***b***	0.60±0.60***b***
Mild glomerular lesions				
Glomerular atrophy	0	3.00±0.00***a***	1.57±0.37***ab***	2.00±0.45***ab***
Hypercellularity	0	0.00±0.00	0.43±0.20***ab***	0.00±0.00***c***
Dilatation of BS	0	0.00±0.00	0.43±0.20***a***	1.00±0.00***abc***
Advanced tubulointerstitial lesions				
Hyaline Cylinders	0	2.86±0.14***a***	1.71±0.28***ab***	2.40±0.24***ab***
Granular Cylinders	0	2.86±0.14***a***	0.71±0.18***ab***	0.40±0.24***ab***
Tubular Calcification	0	3.00±0.00***a***	0.00±0.00***b***	0.20±0.20***b***
Necrosis	0	2.14±0.14***a***	0.00±0.00***b***	0.40±0.40***b***
IFTA	0	3.00±0.00***a***	0.28±0.28***b***	1.20±0.49***ab***
Mild tubulointerstitial lesions				
Tubular Dilatation	0	1.86±0.34***a***	1.71±0.36***a***	1.80±0.49***a***
Interstitial Inflammatory Infiltrate	0	2.00±0.00***a***	1.28±0.36***a***	1.60±0.40***a***
Hidropic Tubular Degeneration	0	----	1.14±0.39***a***	0.80±0.58
Vacuolar TubularDegeneration	0	----	0.00±0.00	0.60±0.40
Vascular lesions				
Arteriolosclerosis	0	2.00±0.00***a***	0.28±0.28***b***	0.40±0.24***b***
Arteriosclerosis	0	1.00±0.00***a***	0.57±0.30***a***	0.60±0.24***a***

Results are presented as Mean ± SEM. a $p<0.05$ vs Sham group; b $p<0.05$ vs CRF group; c $p<0.05$ vs CRF200 group (Mann-Whitney test). BS - Bowman's Space; GBM - glomerular basement membrane; IFTA - interstitial fibrosis and tubular atrophy.

3.5. Renal and liver protein expression

The CRF200NR group presented an increased renal expression of HIF-1α (Fig. 2B1 – 2B5). A significant reduction in tissue renal expression of HIF-2α was observed in the CRF and CRF200NR groups, when compared to Sham group, while no protein expression was found in the CRF200 group (Fig. 2C1 – 2C5). Regarding renal tissue expression of EPO, we found that the CRF group presented a significant reduction in EPO expression, when compared to Sham group, whereas no protein expression was found in the CRF200 and CRF200NR groups (Fig. 2D1 – 2D5).

Significantly increased protein levels of EPO and EPOR were found in the liver of CRF and CRF200NR groups, when compared to Sham (Fig. 3A1and 3A2).

3.6. Expression of kidney inflammatory and fibrosis markers

A significantly higher nuclear expression of NF-κB in renal cortex (Fig. 4A1 – 4A5) and medulla (Fig. 4B1 – 4B5) was found in the CRF and CRF200NR groups, when compared to Sham and CRF200 groups, and similar changes were found for the renal tissue expression of TGF-β1 (Fig. 4C1 – 4C5). Renal IL-6 mRNA expression was significantly up-regulated in the CRF and CRF200NR rats, whereas a significant down-regulation was observed in CRF200 animals (Fig. 4D1). CTGF mRNA levels were significantly up-regulated in all CRF groups, presenting the CRF200NR the highest value (Fig. 4D2). Overexpression of IL-1β was found in the CRF and CRF200NR groups, when compared to Sham and CRF200 groups; the CRF200 group presented a significantly reduced kidney IL-1β levels (Fig. 4E).

4. Discussion

The correction of CKD-associated anemia can be achieved by the use of ESA [10]. Most of the patients respond to this therapy, however, 5-10% of CKD patients become hyporesponsive, presenting worsening of anemia and a poorer outcome [12]. ESA hyporesponse is well documented by several observational and clinical studies in humans; however, the cellular and molecular mechanisms underlying remain to be clarified.

In this study, using the 5/6 nephrectomy-induced remnant kidney rat model of CKD-associated anemia under rHuEPO therapy, we found that a group of rats (CRF200NR), after an initial adequate response to rHuEPO (with increased RBC count, Hb concentration, Ht and reticulocytes) developed hyporesponsiveness to rHuEPO in the two following weeks of treatment, as showed by the significantly reduced values of RBC count, Hb concentration and Ht, at the end of protocol (Table 2). This group presented a hyporegenerative anemia at the 3rd week, with a poor erythropoietic response, as reflected by the RPI and E:M ratio that were significantly reduced (Table 2). The CRF group also presented anemia, showing a reduction in reticulocytes and in RPI to values that were, however, similar to those found for Sham and CRF200 groups, indicating, therefore, some response to anemia. As an attempt to compensate anemia, both CRF and CRF200NR groups presented increased serum EPO levels, due, in part, to increased liver EPO production (Fig. 3A1) [24,25] that appears to be inadequate to overcome anemia [23,26,27]. Considering that the CRF200NR group was under treatment with the same rHuEPO dose, and the endogenous (hepatic) EPO was increased, it would be expected to find an increase in RPI and E:M ratio, but none of this was found, suggesting a blockade to EPO stimuli. Antibodies anti-rHuEPO could also be a cause for ESA hyporesponsiveness [16]; however, in a previous study conducted by our group, we found that until 3 weeks of rHuEPO treatment no antibodies anti-rHuEPO were

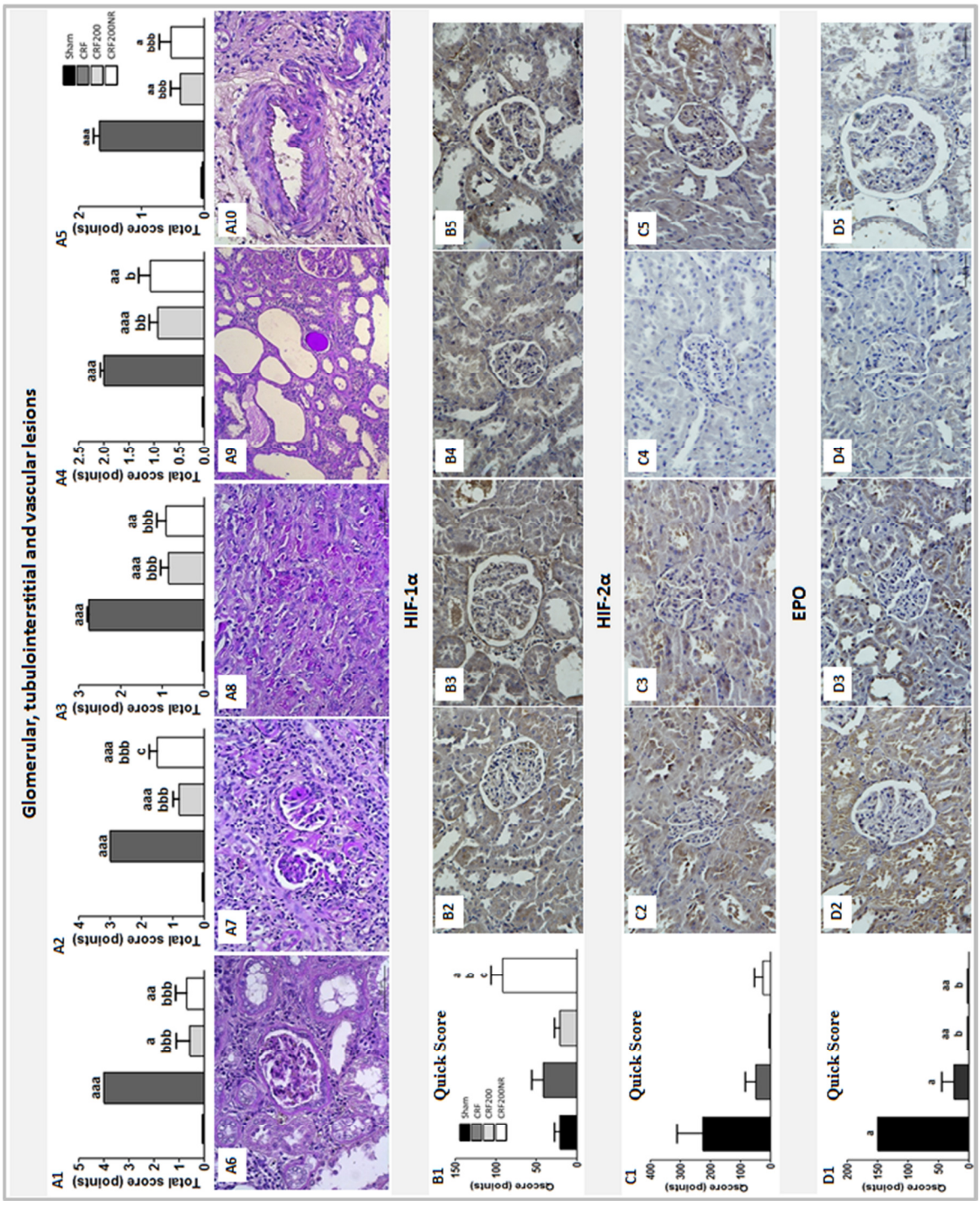
Glomerular, tubulointerstitial and vascular lesions
HIF-1α
HIF-2α
EPO
Quick Score
Sham
CRF
CRF200
CRF200NR
Total score (points)
Qscore (points)

Fig. 2 – Glomerular, tubulointerstitial and vascular lesions and immunostaining of hypoxia inducible factor 1 alfa (HIF-1α), HIF-2α and erythropoietin (EPO) in kidney. Total score and representative lesions observed in kidneys of rat groups under study, at the final time (PAS staining): A1 – total score for advanced glomerular lesions; A2 - total score for mild glomerular lesions; A3 - total score for advanced tubulointerstitial lesions; A4 - total score for mild tubulointerstitial lesions; A5 - total score for vascular lesions; A6 – glomerulosclerosis; A7 - glomerular atrophy; A8 – tubular necrosis and interstitial fibrosis and tubular atrophy (IFTA); A9 – tubular dilations, hyaline cylinders and - interstitial inflammatory infiltrate; A10 – arteriolosclerosis. The expression of HIF-1α, HIF-2α and EPO were not present in the glomerulus or renal medulla tubules (negative reaction). B1 - quick score for HIF-1α; B2 – HIF-1α immunostaining Sham group: light positive cytoplasmic immunoreactivity in convoluted tubules (CT); B3 - HIF-1α immunostaining CRF group: moderate positive cytoplasmic immunoreactivity in CT; B4 - HIF-1α immunostaining CRF200 group: light positive cytoplasmic immunoreactivity in CT; B5 – HIF-1α immunostaining CRF200NR group: moderate positive cytoplasmic immunoreactivity in CT; C1 - quick score for HIF-2α; C2 – HIF-2α immunostaining Sham group: moderate positive nuclear and cytoplasmic immunoreactivity in CT; C3 - HIF-2α immunostaining CRF group: light positive nuclear and cytoplasmic immunoreactivity in CT; C4 - HIF-2α immunostaining CRF200 group: negative reaction; C5 – CRF200NR group: light positive nuclear and cytoplasmic immunoreactivity in CT; D1 - quick score for EPO; D2 – EPO immunostaining Sham group: moderate positive cytoplasmic immunoreactivity in CT; D3 - EPO immunostaining CRF group: light positive cytoplasmic immunoreactivity; D4 and D5 - EPO immunostaining CRF200 and CRF200NR groups: negative reaction. Results are presented as mean ± SEM. a $p<0.05$, aa $p<0.01$, aaa $p<0.001$ vs Sham group, b $p<0.05$, bb $p<0.01$, bbb $p<0.001$ vs CRF group, c $p<0.05$ vs CRF200 group (Mann-Whitney test).

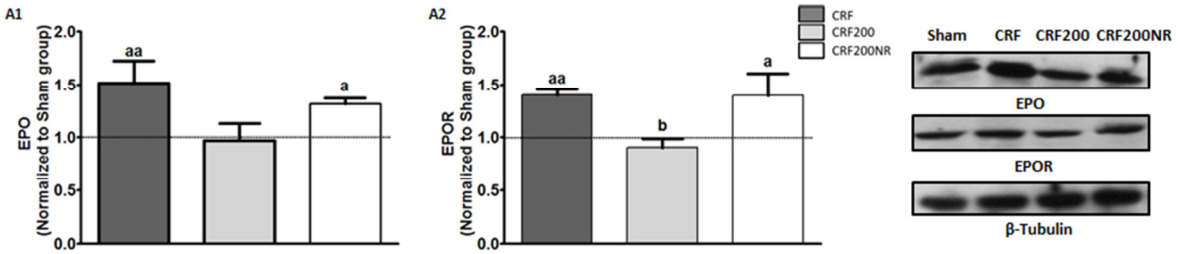

Fig. 3 – Evaluation of liver proteins by western blotting and representative image of western blot for the different groups. A1 – EPO; A2 - EPO receptor (EPOR). Results are presented as mean ± SEM. a $p<0.05$, aa $p<0.01$ vs Sham group, b $p<0.05$ vs CRF group (Mann-Whitney Test).

detected in rats serum [28]. In the case of CRF group the reduced renal tissue is unable to increase EPO levels required to correct anemia.

Inflammation has been associated with ESA hyporesponsiveness [29,30]. Inflammation can lead to ESA hyporesponsiveness, due to the inhibitory effects of inflammatory cytokines on erythropoiesis, on EPO production, and by reducing disturbances in iron metabolism. Several cytokines (such as tumor necrosis factor alpha [TNF-α], IL-1β and interferon gamma [IFN-γ]) induce erythropoiesis suppression, through a direct action on erythroid progenitors [32]. We found that serum CRP levels were increased in all CRF groups, presenting CRF and CRF200NR the highest CRP values (Table 3), consistent with the presence of higher inflammatory condition. It was

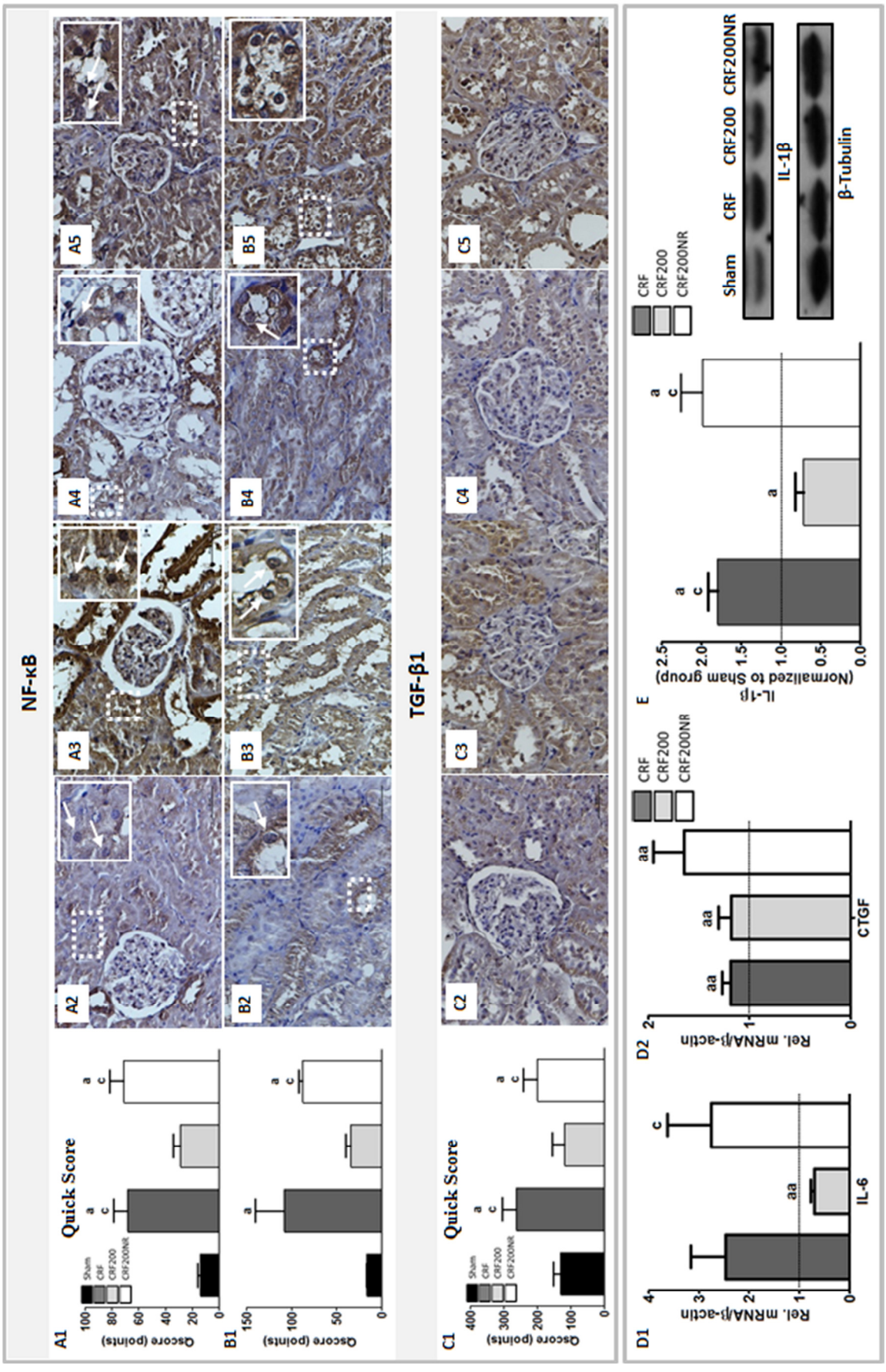
NF-κB
TGF-β1
A1
A2
A3
A4
A5
B1
B2
B3
B4
B5
C1
C2
C3
C4
C5
Quick Score
Qscore (points)
Sham
CRF
CRF200
CRF200NR
D1
D2
E
Rel. mRNA/β-actin
IL-6
CTGF
IL-1β
(Normalized to Sham group)
Sham
CRF
CRF200
CRF200NR
IL-1β
β-Tubulin

Fig. 4 – Immunostaining of nuclear factor kappa B (NF-κB) and transforming growth factor beta 1 (TGF-β1) in kidney. For NF-κB only nuclear staining were considered for analysis A1 - quick score for NF-κB in renal cortex; A2 - NF-κB immunostaining Sham group: moderate positive nuclear immunoreactivity in convulated tubules (CT); A3 - NF-κB immunostaining CRF group: intense positive nuclear immunoreactivity in glomerulus and CT; A4 - NF-κB immunostaining CRF200 group: moderate positive nuclear immunoreactivity in CT; A5 - NF-κB immunostaining CRF200NR group: : intense positive nuclear immunoreactivity in glomerulus and CT; B_1 – quick score for NF-κB in renal medulla. B2 - NF-κB immunostaining Sham group: moderate positive nuclear immunoreactivity in renal medulla tubules; B3 - NF-κB immunostaining CRF group: intense positive nuclear immunoreactivity in renal medulla tubules; B4 - NF-κB immunostaining CRF200 group: moderate positive nuclear immunoreactivity in renal medulla tubules; B5 - NF-κB immunostaining CRF200NR group: intense positive nuclear immunoreactivity in renal medulla tubules; C1 - quick score for TGF-β1 in renal cortex; C2 – TGF-β1 immunostaining Sham group: light positive cytoplasmic immunoreactivity in CT; C3 – TGF-β1 immunostaining CRF group: intense positive cytoplasmic immunoreactivity in CT, glomerulus and interstitial cells; C4 – TGF-β1 immunostaining CRF200 group: moderate positive cytoplasmic immunoreactivity in CT; C5 – TGF-β1 immunostaining CRF200NR group: intense positive cytoplasmic immunoreactivity in CT, glomerulus and interstitial cells. Kidney relative mRNA expression of inflammatory and fibrosis related genes, with B-actin as the reference gene. D1 - interleukin 6 (IL6): D2 - connective tissue growth factor (CTGF); E – Kidney western blot analysis of IL-1β and representative image of western blot for the different groups. Results are presented as mean ± SEM: a $p<0.05$, aa $p<0.01$ vs Sham group; c $p<0.05$ vs CRF200 group (Mann-Whitney test).

recently reported that a minor increase of CRP values in CKD patients increases the risk for development of ESA hyporesponsiveness [31]. In addition, CRF and CRF200NR groups also showed high levels of renal inflammatory markers, such as IL-6 and IL-1β (Fig. 4D1 and 4E, respectively). The pro-inflammatory cytokines, IL-1β and TNF-α, expressed in a NF-κB dependent manner, are known to induce EPO suppression [33,34]. This is in line with our data as in CRF and CRF200NR groups we found an increased renal expression of NF-κB, IL-1β and IL-6 (Fig. 4); moreover, we observed a reduced renal EPO expression in the CRF group and no renal EPO expression in the CRF200NR (Fig. 2). Under inflammatory conditions, the activation of inflammatory cells lead to the production of oxygen metabolites that are able to reduce RBC life span, by inducing metabolic changes and RBC membrane injuries [35]. The reduced glomerular filtration rate (GFR) in CKD leads to accumulation of high levels of uremic toxins that induce metabolic and morphological RBC changes and are pro-inflammatory inducers; thus, uremic toxins may contribute to enhance the oxidative stress, RBC removal, inhibition of erythropoiesis, favoring ESA hyporesponsiveness [36]. We found significantly increased serum creatinine levels in the CRF200NR group when compared to the CRF and CRF200 groups (Table 1), reflecting a reduced GFR and, thus, increased levels of uremic toxins that might contribute to rHuEPO hyporesponsiveness.

Inflammation and uremic toxins may also induce a reduction in renal EPO production [5]. Uremic toxins, by enhancing oxidative stress, aggravate local hypoxia in renal tubular cells, promote disturbances in the oxygen (O_2) sensing system and in EPO production [37].

Erythropoiesis and iron metabolism are closely linked, as increased erythropoiesis leads to an increase in iron absorption and mobilization, in order to meet bone marrow requirements. It has been reported that CKD patients under dialysis and ESA therapies present inflammation and high hepcidin levels, leading to a functional iron deficiency that is enhanced in the non-responders to rHuEPO [38]. In our study, in spite of the evidences of an inflammatory condition in CRF group, no significant changes were found in iron metabolism, hepcidin expression was down-regulated, and the expression of the factors responsible for hepcidin up-regulation was similar versus Sham group, except TfR1 that was up-regulated (Fig. 1). In fact, we found a down-regulation of hepcidin expression in CRF and CRF200NR groups. Hepcidin expression is regulated by the balance between several factors, including inflammation, liver iron, TSAT (stimulators of hepcidin synthesis), hypoxia and erythropoiesis (inhibitors of hepcidin synthesis) [19]. In CRF rats, hypoxia and anemia appears to have a stronger influence on hepcidin expression than inflammation. The down-regulation of hepcidin in CRF200NR rats seems to be due to other inhibitory factors, namely to the down-regulation of TfR1, HJV and BMP6. Moreover, rHuEPO has an inhibitory effect on hepcidin expression [38,39].

Inflammation and anemia are linked to progression of renal disease and are both enhanced in non-responders to ESA therapy [15,17]. In the kidney, hypoxia leads to activation of the HIF system, stimulating several target genes [7]. HIF-2α is the main regulator of renal and liver EPO production [40], whereas HIF-1α appears as the main regulator of glycolytic enzymes and of other factors involved in renal fibrosis, such as connective tissue growth factor (CTGF) [41]. Renal fibrosis is the final feature of CKD progression, characterized by tubular atrophy, increased deposition of extracellular matrix (ECM) and alterations in renal blood vessels [9]. The initial (acute) activation of HIF-1α is a protective mechanism triggered to achieve correction of anemia/hypoxia; however, a continuous activation promotes several renal lesions, leading to renal fibrosis and tissue injury. CTGF is a marker of renal fibrosis, as it increases the production of ECM, fibroblasts cell growth and TGF-β1 activity [42,43]. CTGF and TGF-β1 have similar effects, leading to renal fibrosis. TGF-β1 also promotes glomerulosclerosis and presents immunomodulatory effects [44]. Indeed, TGF-β1 released by injured renal cells acts as an anti-inflammatory agent, through the recruitment of monocytes; however, its continuous expression leads to a pathological accumulation of ECM and release of excessive inflammatory cytokines, such as IL-1β and IL-6, through a NF-κB-dependent manner, thus promoting fibrosis [44]. These enhanced continuous response mechanisms create a vicious cycle where inflammation promotes hypoxia and vice-versa; moreover, both conditions contribute to increase the expression of NF-κB, which appears to directly regulate HIF-1α expression [45]. The development of anemia (Fig. 5) in the rHuEPO hyporesponsive group (CRF200NR), associated with uremia and a systemic and renal inflammatory state, by favoring renal hypoxia, trigger an increase in renal expression of HIF-1α, TGF-β1 and CTGF, that will further aggravate renal fibrosis (showed by the increased score of IFTA). The renal fibrosis, by inducing an inflammatory response, creates a vicious cycle that promotes CKD progression (Fig. 5). The CRF and the CRF200NR groups presented similar inflammatory and fibrosis features. The more advanced renal lesions of CRF rats may explain the reduced expression of the HIF system, due to the reduced renal capacity to induce HIF activation [46].

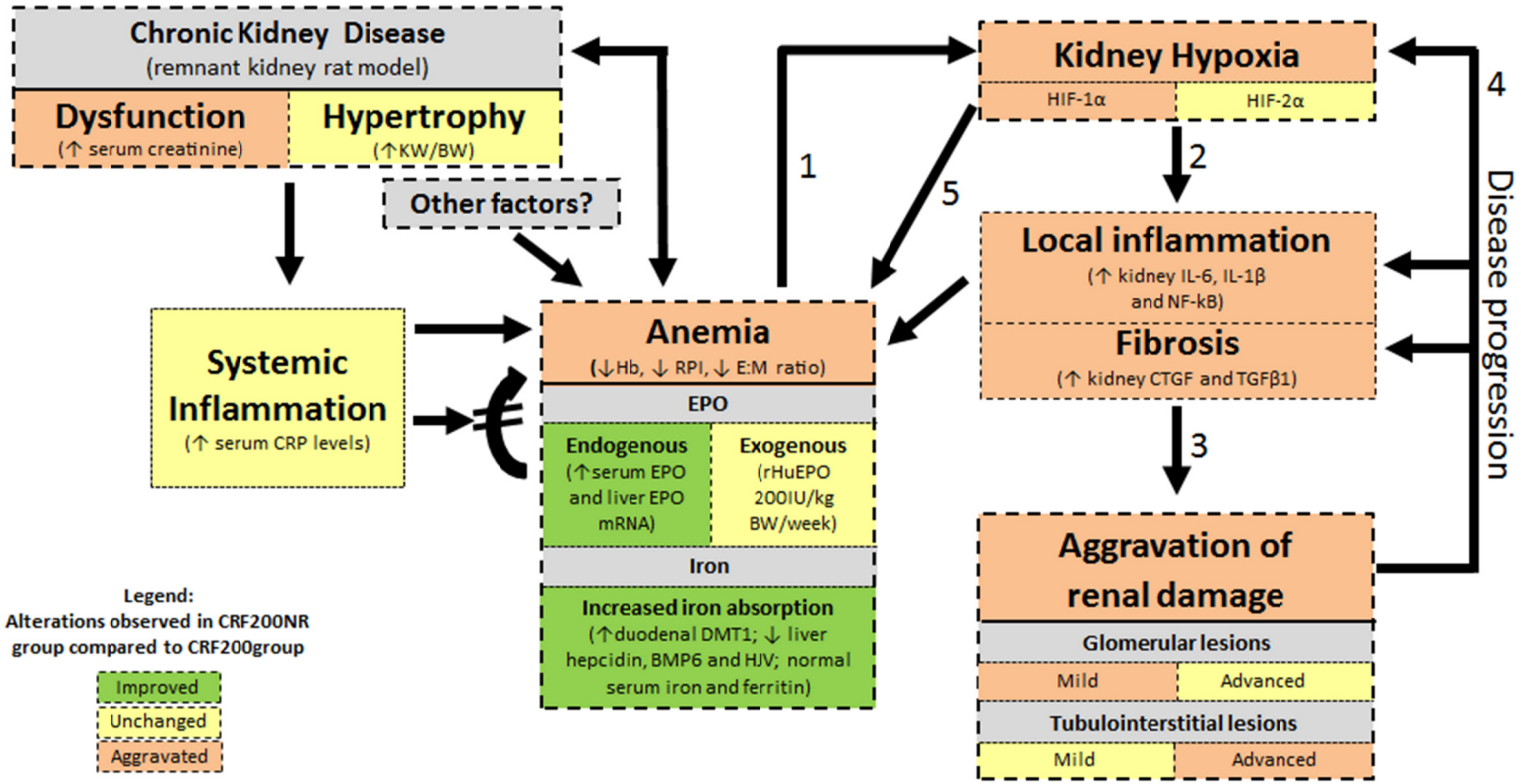

Fig. 5 – Schematic diagram of proposed mechanisms underlying recombinant human erythropoietin (rHuEPO) hyporesponsiveness in chronic kidney disease (CKD) - CRF200NR vs CRF200 at 3rd week. Systemic and local inflammation seems to contribute to anemia of CKD due to its effects on red blood cells and to the blunting of erythropoietin (EPO) action. Anemia is accompanied by increased serum endogenous EPO levels and augmented iron absorption. (1) Anemia induces kidney hypoxia, leading to HIF-1α increase, (2) which promotes fibrosis and exacerbation of local inflammation, (3) with consequent aggravation of renal damage, (4) contributing to the progression of renal disease. The progression of CKD increases kidney hypoxia, local inflammation and fibrosis, (5) aggravating anemia. BMP6 – bone morphogenetic protein 6; CRP – C-reactive protein levels; CTGF - connective tissue growth factor; DMT1 - divalent metal transporter 1; E:M – erythroid:myeloid; Hb – hemoglobin; HIF – hypoxia inducible factor; HJV – hemojuvelin; IL – interleukin; KW/BW – kidney weight/body weight ratio; NF-kB - nuclear factor kappa B ; RPI – reticulocyte production índex; TGFβ1 - transforming growth factor 1.

It is known that rHuEPO stimulates erythropoiesis and has also pleiotropic properties, namely anti-inflammatory and renoprotective effects, among others [11]. Indeed, we found that both CRF groups under rHuEPO treatment presented an improvement in all types of renal lesions, when compared to the CRF group (Fig. 2 and Table 4). The CRF200NR group presented a better renal lesion profile than the CRF group, but worse than the CRF200 animals, presenting higher scores for glomerular atrophy, dilatation of Bowman's Space, hyaline cylinders and IFTA (Table 4); a trend towards a higher KW/BW ratio, indicating a higher level of kidney hypertrophy was also observed (Table 3). In addition, no improvements were found in NF-κB, TGF-β1, IL-6 and IL-1β renal expression, nor in serum CRP levels in the CRF200NR group, when compared to the CRF group. A significant increase in kidney CTGF expression was also found in the CRF200NR group, along with increased HIF-1α levels, when compared to the CRF group. Thus, the existence of anemia/hypoxia, due to the reduced response to rHuEPO, and inflammation seem to blunt the pleiotropic effects of rHuEPO.

In conclusion, our study suggests that inflammation is an important determinant in hyporesponsiveness to rHuEPO (when no major changes in iron metabolism are observed) blunting the response to endogenous (hepatic) EPO and to rHuEPO. This hyporesponse causes the reappearance of anemia that contributes to worsening of renal fibrosis and to the progression of CKD, thus explaining the poorer outcome of non-responders CKD patients. Our data suggest that a therapeutic approach able to reduce inflammation in CKD patients could improve the response to ESA therapy and reduce premature mortality associated with that condition.

Conflict of Interest

No relevant conflicts of interest to disclose.

Acknowledgments

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Paper IV

Impaired renal endothelial nitric oxide synthase and reticulocyte production as modulators of hypertension induced by recombinant human erythropoietin in the rat

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Impaired renal endothelial nitric oxide synthase and reticulocyte production as modulators of hypertension induced by recombinant human erythropoietin in the rat

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ABSTRACT

Our aim was to study the effect of a broad range of recombinant human erythropoietin (rHuEPO) doses on hematological and biochemical parameters, blood pressure (BP) and renal function and damage in the rat, focusing on endothelial nitric oxide synthase (eNOS) and hypoxia-inducible factors (HIFs).

Male Wistar rats were divided in 5 groups receiving different doses of rHuEPO (100, 200, 400 and 600 IU/kg body weight (BW)/week) and saline solution (control), during 3 weeks. Blood and 24h urine were collected to perform hematological and biochemical analysis. BP was measured by the tail-cuff method. The kidney tissue was collected to mRNA and protein expression assays and to characterize renal lesions.

A dose-dependent increase in red blood cells count, hematocrit and hemoglobin levels was found with rHuEPO therapy, in rHuEPO200, rHuEPO400 and rHuEPO600 groups. Increased reticulocyte count was found in the rHuEPO400 and rHuEPO600 groups. BP raised in all groups receiving rHuEPO. The rHuEPO200 and rHuEPO600 groups presented increased kidney protein levels of HIF2α and a reduction in kidney protein levels of eNOS, along with the highest grade of vascular and tubular renal lesions.

Our study showed that rHuEPO-induced hypertension is present before significant hematological changes and, therefore, might involve indirect (hematological) and direct (renal) effects which varies according to the dose used. The presence of renal hypoxia reduces eNOS activity. Excessive erythrocytosis increases blood hyperviscosity which can be modulated by an increase in reticulocytes, reducing renal hypoxia. Hypertension leads to early renal damage without alterations in traditional markers of renal function, thus masking the serious adverse effects and risks.

Keywords: direct renal vascular effects; endothelial nitric oxide synthase; hypertension; hypoxia; recombinant human erythropoietin therapy; reticulocytes.

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1. Introduction

The main clinical indication of recombinant human erythropoietin (rHuEPO) is the correction of anemia in chronic kidney disease (CKD) and cancer patients [1], but the discovery of non-hematopoietic actions of rHuEPO [2, 3] increased the number of patients that could putatively benefit from this therapy [4-6]; however, to achieve those effects higher doses are usually needed, which increase the risk and incidence of adverse events [7]. Several studies reported that CKD patients treated with higher rHuEPO doses present a higher mortality rate and increased cardiovascular events [8, 9]. In addition, clinical trials using rHuEPO for the treatment of acute ischemic stroke showed increased mortality rates in patients under high rHuEPO doses [7, 10]. Hypertension is a common side-effect associated with rHuEPO therapy [11, 12], as previously reported in predialysis and hemodialysis patients [11, 13, 14]. The same effect was also demonstrated in healthy subjects under rHuEPO therapy with prolonged low-dose treatments or with short-term administration of high doses [15, 16]. This situation is particularly applicable in illicit rHuEPO use in sports, as doping, raising serious concerns about the potential adverse effects on athletes, as we and others previously reported in animal models and in human studies [17, 18].

Several mechanisms have been proposed to explain the hypertension associated with rHuEPO therapy [13]. rHuEPO-stimulated erythropoiesis increases the hematocrit (Ht), thus causing an increase in blood viscosity, with a consequent increase in vascular resistance and blood pressure (BP). The rHuEPO-evoked increase in hemoglobin (Hb) and Ht levels are the result of red blood cell (RBC) mass increase and plasma volume reduction [19]. However, these effects seem to be unable to entirely explain the hypertension observed with this therapy; in fact, previous studies has showed that rHuEPO might directly act on both endothelial and vascular smooth muscle cells (VSMC), promoting a disequilibrium between vasodilators and vasoconstrictors [10].

The kidney plays a major role in BP control due to its capacity to excrete sufficient sodium and water to regulate the extracellular fluid and the blood volume [20]. Renal disease and hypertension are strictly linked in such a way that each could be the cause or the consequence of the other [21]. In fact, hypertension impairs renal microvasculature, compromising kidney function, promoting hypoxia and evolution of damage, which is exacerbated by the disequilibrium between vasodilators and vasoconstrictors [12]. Despite the evidence of rHuEPO-evoked BP raise, independently of hematological actions, the contribution of renal vasculature impairment and kidney hypoxia/damage to the pro-hypertensive properties of rHuEPO remain to be elucidated.

In this work we aimed to study the effect of increasing doses of rHuEPO on hematological and biochemical parameters, and to clarify mechanisms associated with BP increase.

2. Animals and methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab. Inc., Chatillon-sur-Chalaronne, France), with 320-350 g of body weight (BW), were maintained in ventilated cages, in an air conditioned room, subjected to 12 h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. Animal experiments were conducted according to the European Community Council directives on animal care and to the national authorities. The rats were randomly divided in five groups (7-8 rats each group) receiving a subcutaneous (sc) injection of saline solution (control group) or rHuEpo (NeoRecormon®, Roche) 100, 200, 400 or 600 IU/kg BW/week (rHuEPO100, rHuEPO200, rHuEPO400

and rHuEPO600 groups, respectively), 3 times per week, during 3 weeks.

Systolic, diastolic and mean blood pressure (SBP, DBP and MBP) measures were obtained by the tail-cuff method, using a sphygmomanometer (LE5001 Pressure meter, Panlab Havard Apparatus, Barcelona, Spain) in appropriate contention cages, according to previously described [22]. In brief, before the measurements, the rats were warmed for 10-20 min at 25-30 ºC in order to allow the detection of tail artery pulsations and to achieve the pulse level ready. BP and HR values were obtained averaging 8-10 measurements of two consecutive days. To minimize stress-induced fluctuations in BP, all the rats were adapted to appropriate cages and to measurements during at least 2 weeks before the beginning of measurements. The same person took the final values in the same peaceful environment between 2:00 and 6:00 pm.

2.2. Sample collection

Blood samples were collected, at the end of protocol, with rats under anesthesia (intraperitoneal) with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncuture, from the jugular vein, into Vacuette® tubes without anticoagulant (to obtain serum) or with K_3EDTA (to obtain plasma) for hematological and biochemical studies. Aliquots were immediately stored at -80ºC until assayed.

For collection of 24h urine the rats were enclosure, at the end of protocol, in metabolic cages during 24h with free access to laboratory chow and tap water. Afterwards, urine volume and water consumption were recorded. Aliquots of urine were made and stored at -80ºC.

At the end of protocol, after blood collection and under anesthesia, the rats were sacrificed by cervical dislocation; kidneys were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighted. In order to isolate total RNA, 0.2 g samples of kidney, from each rat, were immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) upon collection and stored at 4°C for 24h; afterwards, samples were frozen at -20ºC. For western blot analysis organs were immediately frozen with liquid nitrogen and stored at -80ºC.

2.3. Biochemical and hematological assays

Serum creatinine, blood urea nitrogen (BUN) and uric acid were analyzed through automatic methods and equipment (Hitachi 717 Chemistry Analyzer, Roche Diagnostics, Basel, Switzerland).
RBC count, Ht, Hb, mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were assessed in whole blood K_3EDTA using an automated counter (HORIBA ABX, Amadora, Portugal). Reticulocyte count was measured by microscopic counting on blood smears after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA). Briefly, after blood collection into K_3EDTA tubes and 10 min of homogenization, 25 µL of blood were added to 25 µL of reticulocyte stain (New methylene blue). After 20 min of rest, 2 blood smears from each rat were made.

Serum levels of vascular endothelial growth factor (VEGF) were evaluated by rat specific ELISA kit, according to manufacture instructions (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA).

The urinary levels of urea, creatinine, uric acid and albumin were analyzed using automatic methods (Cobas Integra 400Plus, Roche Diagnostics, Basel, Switzerland). Creatinine clearance and glomerular

filtration rate (GFR) were calculated according to Pestel et al.[23].

2.4. Gene expression analysis

Kidney RNA isolation and integrity control were performed as previously described by us [24]. One microgram of total RNA was reversely transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer instructions. One nanogram of cDNA was used for gene expression analysis with qPCR using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs (Table 1). qPCR reactions are performed using the following conditions: enzyme activation at 95ºC for 40s; denaturation at 95ºC for 3s; annealing for 30s (for each pair primer temperature refer to Table 1). Gene expression was normalized to beta actin (a*ctb*) and alpha tubulin (*tuba*), and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. The following genes expression was analyzed in the kidney: *arnt* (aryl hydrocarbon receptor nuclear translocator; HIF-1β); *arnt2* (aryl hydrocarbon receptor nuclear translocator 2; HIF-2β); *epas1* (endothelial PAS domain protein 1; HIF-2α); e*po* (erythropoietin); *hif1α* (eypoxia-inducible factor 1, alpha subunit) and *vegfα* (vascular endothelial growth factor alpha; VEGFα).

2.5. Western Blot

Kidney proteins were extracted using RIPA buffer (NaCl 150mM, Tris-HCl 50mM pH8, Triton X-100 1%, ethylene glycol tetraacetic acid 5mM, deoxycholic acid 0.5%, Sodium lauryl sulfate 0.1%) and ultra-sonication. After centrifugation, protein concentration in supernatant was assayed using the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, IL, USA). Aliquots of the extract containing 100 µg of protein were separated by reducing

Table 1 - List of primer sequences and annealing temperatures

Gene	Primer sequences (5' → 3')	Annealing temperature
actb	F: TACAGCTTCACCACCACAGC R: AAGGAAGGCTGGAAGAGAGC	57ºC
arnt	F: AGAATGGCTGTGGATGAG R: GGTTCCTGGCTAGAGTTC	56ºC
arnt2	F: TGAAAGAAGGAGAAGCCCAATA R: CATCAGAGTTATGCCGAGACAG	58ºC
epas1	F: TGACTTCACTCATCCTTGCGACCA R: ATTCATAGGCAGAGCGGCCAAGTA	59ºC
epo	F: TCTGACTGACCGCGTTACTC R: GCCCAGAGGAATCAGTAGCA	59ºC
hif1a	F: CTCACCATCAGTTACTTAC R: GTCACCATCATCTGTTAG	58ºC
tuba	F: CAC CCG TCT TCA GGG CTT CTT GGT TT R: CAT TTC ACC ATC TGG TTG GCT GGC TC	59ºC
vegfa	F: GAAGTTCATGGACGTCTACCAG R: CATCTGCTATGCTGCAGGAAGCT	58ºC

F: Forward; R: Reverse; *actb* – beta actin; a*rnt* - aryl hydrocarbon receptor nuclear translocator; a*rnt2* - aryl hydrocarbon receptor nuclear translocator 2; e*pas1*- endothelial PAS domain protein 1; *epo*- erythropoietin; *hif1a*- hypoxia-inducible factor 1, alpha subunit; *tuba* – alfa tubulin; *vegfa* - vascular endothelial growth factor alpha.

SDS-PAGE (10 %) and transferred onto nitrocellulose membranes. The membranes were blocked using 7% non-fat milk in a solution of Tris-buffered salt with Tween-20. The nitrocellulose membranes were incubated, overnight at 4ºC, with rabbit anti-EPAS-1 (HIF2α) antibody 1:200 (sc- sc-28706), rabbit anti-IL-3/IL-5/GM-CSFRβ antibody (β common receptor) 1:100 (sc-678), mouse anti-ERK1 1:200 (sc-37685), rabbit anti-phospho-ERK1/2 1:100 (sc-16982), rabbit-anti AKT1/2/3 1:150 (sc-8312, Santa Cruz Biotechnoloy, Inc, Texas, USA), rabbit anti-phospho AKT1/2/3 1:250 (ab81283), rabbit anti-STAT5 1:250 (ab32043), rabbit anti-phospho-STAT5 1:250 (ab30648), mouse anti-phospho eNOS 1:500 (612393) and mouse anti-eNOS 1:500 (610297, BD Biosciences, New Jersey, USA); afterwards, they were incubated with anti-mouse (A9044) or anti-rabbit (sc-2004) secondary

antibody-conjugated with horseradish peroxide (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA; Santa Cruz Biotechnoloy, Inc, Texas, USA, respectively). Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL WesternBright, Advansta, California, USA). The analysis of the immunoblots was performed by densitometry (Bio1D++ version 99, Vilber Lourmat). To ensure even loading of the samples, all immunoblots were probed with rabbit anti-β-tubulin antibody 1:500 (sc-9104, SantaCruz Biotechnology, Texas, USA). The protein concentration in each sample was normalized for Control group.

2.6. Histopathological analysis

Samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3μm thick sections were stained with Periodic acid of Shiff (PAS). All samples were examined by light microscopy using a Microscope Zeiss Axioplan 2 and images were captured using a digital microscope camera (Leica DFC450). Lesions were evaluated on the total tissue on the slide in a double-blinded fashion by the pathologist, who evaluated and quantified lesions according to previously described by us [24]. Briefly, glomerular damage was assessed by evaluating mesangial expansion, nodular sclerosis, glomerulosclerosis, glomerular basement membrane, capsule of Bowman thickening, glomerular atrophy or hypertrophy and hyalinosis of the vascular pole. A semi-quantitative rating for each slide ranging from normal (or minimal) to severe (extensive damage) was assigned to each component. Severity was graded as absent/normal, mild, moderate, and severe. Scoring was defined according to the extension occupied by the lesion (5% area): normal <25%; mild 25-50%; moderate 50-75%; severe >75%. The final score of each sample was obtained by the average of score observed in individual glomeruli in the considered microscopic fields. Glomerular hypertrophy was analysed by measuring the area of ten glomeruli in cortical area and ten juxtamedullary glomeruli, selected by unbiased method, in each rat, using for analysis the ImageJ processing software (National Institutes of Health, Maryland, USA). Analysed tubulointerstitial lesions comprised interstitial inflammatory infiltration, presence of hyaline and granular cylinders, hidropic and vacuolar degeneration, and the association of interstitial fibrosis and tubular atrophy (IFTA). Tubulointerstitial damage was evaluated and graded by the same semi-quantitative method used in glomerular analysis, with the exception of IFTA, which was graded as normal, if absent, as mild, moderate, and severe, if present in <25%, between 25-50%, and over 50% of affected area, respectively. The evaluation of vascular lesions was concentrated on arteriolosclerosis and arteriosclerosis. Arteriolosclerosis was scored as 0 if absent, as 1 if one arteriole with arteriolosclerosis was present, and as 2 if more than one arteriole with arteriolosclerosis was observed in the entire slide. Arteriosclerosis was scored as 0 if no intimal thickening was present, as 1 if intimal thickening was less than the thickness of media, and as 2 if intimal thickening was more than the thickness of the media and considering the worst artery on the slide.

2.7. Statistical analysis

Results are presented as mean ± standard error mean (SEM). For comparison between groups Mann-Whitney test was performed. The strength of the association between the variables vascular and tubular lesions, SBP, hematological (RBC, Hb, Ht and reticulocytes) and biochemical (serum BUN and creatinine, creatinine clearance and GFR) markers was estimated by Spearman's correlation coefficient. Statistical significance was accepted at $p<0.05$. Statistical analysis was performed using the IBM Statis-

tical Package for Social Sciences (SPSS) for Windows, version 22.0 (IBM, New York, USA).

3. Results

We found a significant dose-dependent increase in RBC, Ht, Hb and MCV values and a reduction in MCHC for the rHuEPO200, rHuEPO400 and rHuEPO600 groups (Table 2). A significant increase in reticulocyte count was found for the rHuEPO400 and rHuEPO600 groups. The group receiving the lower dose (rHuEPO100) did not show any significant alteration in the hematological parameters, as compared to the control group (Table 2).

The markers of renal function in serum and urine were not significantly altered, with the exception of BUN that was significantly decreased in the rHuEPO400 group and increased in the rHuEPO600 group, both compared to the control (Table 2). The rHuEPO600 group presented significantly increased serum uric acid levels, *versus* rHuEPO100 and rHuEPO400 groups. Increased serum levels of VEGF were found in the rHuEPO200 and rHuEPO600 groups, when compared to all the other groups (Table 2).

SBP, DBP and MBP values were significantly increased in all groups receiving rHuEPO, as compared to the control (Table 2). The rHuEPO200 and rHuEPO600 groups presented significantly higher SBP values, when compared to the rHuEPO100 and rHuEPO400 groups. In the rHuEPO600 group, MBP was significantly higher *versus* the other rHuEPO-treated groups (Table 2).

Kidney gene expression (mRNA levels) of HIF1α and HIF1β was unchanged in all the groups treated with rHuEPO, versus the control (Fig. 1A), except for the rHuEPO400 group that showed a significant decrease versus all the other groups. Regarding HIF2α and HIF2β, mRNA overexpression was found in the rHuEPO200 and rHuEPO600 groups, accompanied by an increase in the VEGF expression in the same groups, together with a significantly lower EPO mRNA expression in all groups, when compared to the control group (Fig. 1A and 1B).

Significant kidney HIF2α protein overexpression was found in the rHuEPO200 and rHuEPO600 groups, when compared to the control, rHuEPO100 and rHuEPO400 groups, showing these last two groups a significant down-expression of HIF2α versus the control (Fig. 2A). The β common receptor protein expression in the kidney was increased in all rHuEPO-treated groups, when compared to the control (Fig. 2B), accompanying by augmented phosphorylated/total STAT5 ratio (Fig. 2D). In addition, phosphorylated/total eNOS ratio was significantly decreased in the rHuEPO200 and rHuEPO600 groups when compared to the control, rHuEPO100 and rHuEPO400 groups, and significantly increased in the rHuEPO100 and rHuEPO400 groups, versus the control (Fig. 2C). The protein phosphorylated/total AKT1/2/3 ratio was increased in all rHuEPO-treated groups, when compared to the control; however, the rHuEPO200 and rHuEPO600 groups presented a significantly reduction *versus* the rHuEPO400 group (Fig. 2E). The phosphorylated/total ERK1/2:ERK1/2 ration (Fig. 2F) was significantly decreased in the rHuEPO100 and rHuEPO400 groups, whereas in the other two groups (rHuEPO200 and rHuEPO600) no significant changes were found when compared to the control. However, the rHuEPO400 group showed significantly increased values when compared to the rHuEPO100 and rHuEPO400 ones.

Glomerular hypertrophy (Fig. 4b) was the only glomerular lesion found in all groups receiving rHuEPO (rHuEPO100: 10050.4±388.7 μm^2;

Table 2 –Hematological, biochemical and blood pressure data at the end of study protocol

Parameters/ Groups	Control	rHuEPO100	rHuEPO200	rHuEPO400	rHuEPO600
Hematological data					
RBC (x 10^{12}/L)	7.99±0.17	8.20±0.13	9.41±0.12***ab***	9.85±0.23***ab***	10.53±0.39***abc***
Hb (g/dL)	14.47±0.19	14.74±0.30	17.06±0.32***ab***	18.14±0.40***ab***	19.60±0.60***abc***
Ht (%)	43.57±0.85	44.89±1.19	53.21±1.16***ab***	57.37±1.20***abc***	66.56±1.03***abcd***
Ret (%)	2.33±0.18	2.63±0.31	1.90±0.27***b***	5.41±0.48***abc***	4.17±0.51***abc***
Ret (x10^9/L)	205.68±16.16	215.01±26.67	179.34±26.07	535.60±52.38***abc***	469.55±31.74***abc***
MCV (fL)	54.50±0.48	54.88±0.35	57.14±0.59***a***	58.29±0.71***ab***	59.86±1.03***abc***
MCHC (g/dL)	33.27±0.35	32.90±0.33	32.10±0.21***a***	31.63±0.1***abc***	31.14±0.36***ab***
Serum biochemical data					
BUN (mg/dL)	21.02±0.33	22.36±0.51	21.70±0.42	18.36±0.38***abc***	23.06±0.70***ad***
Creatinine (mg/dL)	0.37±0.02	0.32±0.02	0.36±0.03	0.39±0.02	0.40±0.02
Uric acid (mg/dL)	0.81±0.06	0.85±0.03	0.93±0.11	0.76±0.05	1.14±0.19***bd***
VEGF (pg/mL)	321.97±29.54	340.35±33.51	826.64±39.17***abd***	306.50±19.61	697.57±68.02***abd***
Urine biochemical data					
Urea (mg/dL)	5280.00±617.13	5950.00±575.39	6525.00±359.94	5457.00±340.07	5400.00±733.03
Creatinine (mg/dL)	83.00±7.16	82.86±5.65	97.50±7.96	90.00±6.17	90.00±15.92
Uric acid (mg/dL)	10.58±0.66	8.43±0.97	11.00±1.47	11.96±1.91	11.88±1.01
AE (mg/L)	3.00±0.33	3.00±0.65	2.00±0.31	3.71±0.84	2.17±0.48
CC(mL/h/rat)	108.75±5.24	113.32±5.38	105.13±8.49	107.16±8.67	108.06±16.00
GFR (mL/h/rat)	108.76±5.27	111.11±6.21	109.34±6.87	117.68±8.83	110.88±16.21
Blood pressure					
SBP (mmHg)	105.20±0.90	119.06±1.12***a***	129.15±0.85***ab***	118.70±1.9***ac***	145.64±1.23***abcd***
DBP (mmHg)	88.38±1.74	104.05±0.92***a***	100.73±1.30***ab***	98.62±2.44***a***	110.43±1.55***abcd***
MBP (mmHg)	89.50±2.31	107.50±0.95***a***	107.31±1.71***a***	103.53±2.05***a***	122.71±1.55***abcd***

Results are presented as mean ± SEM: a p<0.05 vs control group; b p<0.05 vs rHuEPO100 group; c p<0.05 vs rHuEPO200 group; d p<0.05 vs rHuEPO400 group (Mann-Whitney Test). BUN - Blood urea nitrogen; Hb – Hemoglobin; Ht – Hematocrit; DBP - Diastolic blood pressure; EPO - Erythropoietin; GFR - Glomerular filtration rate; MBP - Mean blood pressure; RBC - Red blood cell; Ret – Reticulocytes; SBP - Systolic blood pressure; VEGF - Vascular endothelial growth factor.

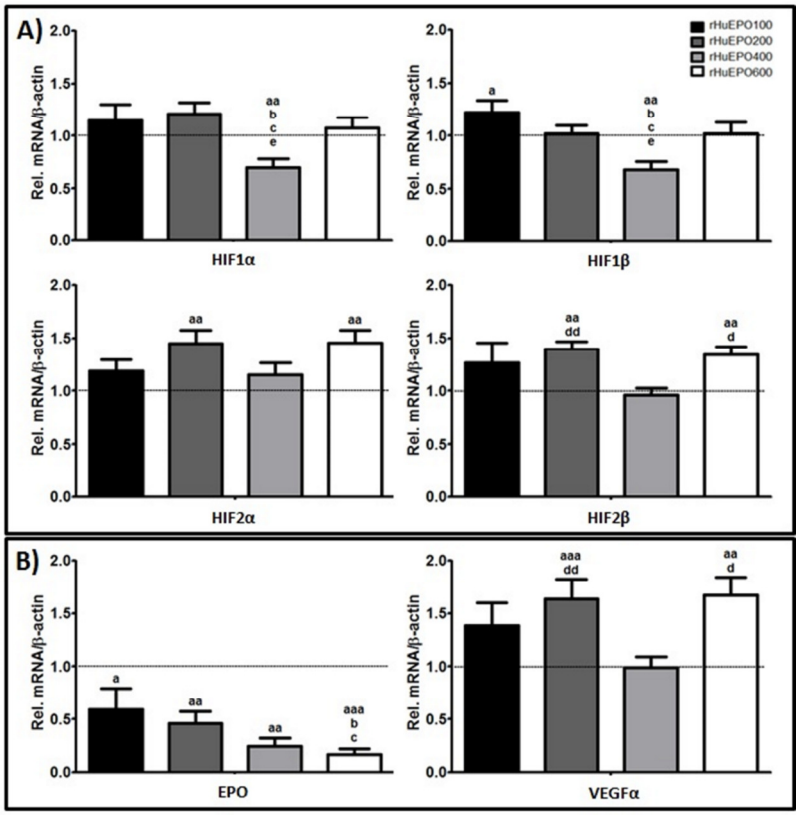

Fig. 1 – Relative mRNA expression of kidney genes. A) hypoxia-inducible factors (HIF) system genes; B) erythropoietin (EPO) and vascular endothelial growth factor alpha (VEGFα) genes. β - actin was used as the reference gene. Results are presented as mean ± SEM: a p<0.05, aa p<0.01, aaa p<0.001 vs control group; b p<0.05 vs rHuEPO100 group; c p<0.05 vs rHuEPO200UI group; d p<0.05, dd p<0.01 vs rHuEPO400 group; e p<0.05 vs rHuEPO600 (Mann-Whitney test).

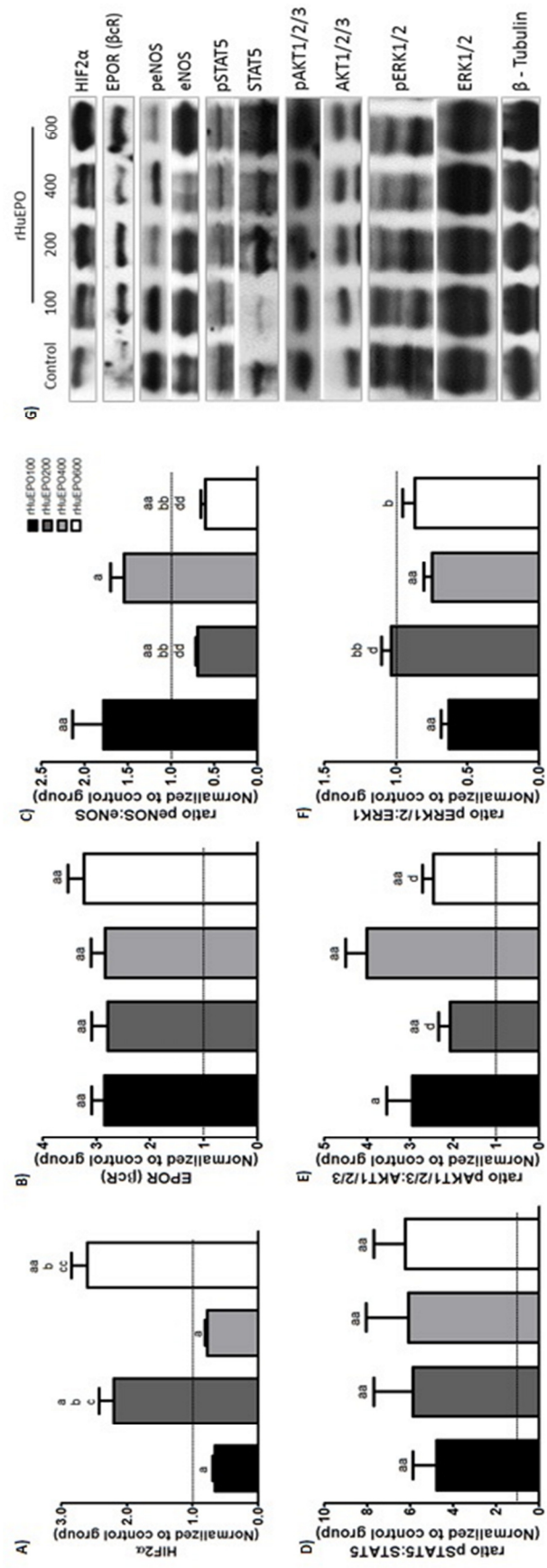

Fig. 2 – Kidney proteins evaluation by Western Blot. A) hypoxia inducible factor alpha (HIF2a); B) erythropoietin receptor (EPOR) - β common receptor, C) ratio phosphorilated endotelial nitric oxide (peNOS) to total eNOS; D) pSTAT5:STAT5 ratio; E) pAKT1/2/3:AKT1/2/3 ratio; F) pERK1/2:ERK1/2 ratio; G) representative image of western blot for the different groups. Results are expressed as mean ± SEM: a $p<0.05$, aa $p<0.01$vs control group; b $p<0.05$, bb $p<0.01$ vs rHuEPO100 group; c $p<0.05$, cc $p<0.01$vs rHuEPO200UI group; d $p<0.05$, dd $p<0.01$vs rHuEPO400 group (Mann-Whitney test

rHuEPO200: 11727.9±363.8 μm^2; rHuEPO400: 10852.4±355.6 μm^2; rHuEPO600: 10888.0±334.8 μm^2 *versus* control: 7950.9±398.7 μm^2, $p<0.05$, Mean±SEM).

Regarding the vascular profile, no lesions were found in the control group, as expected (Fig. 3a and 3b); however, arteriolosclerosis (Fig. 3a and 4d) was found in all rHuEPO-treated groups, with a higher degree in the rHuEPO200, rHuEPO400 and rHuEPO600 groups. Arteriosclerosis (Fig. 3b and 4f) was present in all groups, with statistically significant changes encountered in the rHuEPO200 and rHuEPO600 groups. Hyperemia was encountered in all rHuEPO-treated rats (Fig. 3c and 4h). Additionally, media proliferation in larger arteries (Fig. 4f) and arterioles was observed in all groups. Some degree of neovascularization occurred in all groups receiving rHuEPO (Fig. 4g and 4h). Tubular lesions were found in all groups under rHuEPO therapy, including the presence of hyaline (Fig. 3d and 5a) and granular (Fig. 3e and 5b) cylinders, as well as hidropic (Fig. 3f and 5c) and vacuolar (Fig. 3g and 5d) tubular degeneration. A mild degree of interstitial inflammatory infiltration (Fig. 3h and 5e) was observed in the rHuEPO200 and rHuEPO600 groups, together with a mild degree of IFTA (Fig. 3i and 5f) in the rHuEPO600 group.

We found that tubular lesions correlated positively and significantly with rHuEPO dose ($r=0.422$, $p<0.001$), Ht ($r=0.412$, $p<0.001$), Hb ($r=0.386$, $p<0.001$), SBP ($r=0.337$, $p<0.001$), RBC ($r=0.368$, $p<0.001$), serum creatinine ($r=0.263$, $p=0.004$) and reticulocytes count ($r=0.234$, $p=0.013$).

In addition, vascular lesions correlated positively and significantly with rHuEPO dose ($r=0.497$, $p<0.001$), SBP ($r=0.433$, $p<0.001$), Ht ($r=0.405$, $p=0.001$), Hb ($r=0.404$, $p=0.001$) and RBC ($r=0.382$, $p=0.002$).

4. Discussion

Although several mechanisms has been proposed to explain the rHuEPO-evoked BP raise the issue remains highly debatable, deserving further elucidation [13].

In our study, increased SBP was found in all groups receiving rHuEPO therapy, even in the absence of significant hematological changes, as observed in the rHuEPO100 group, thus suggesting the involvement of others factors. In fact, VSMC within the media layer of arteries can be a direct target to rHuEPO due to promotion of proliferation and contraction, thus leading to increased vascular resistance [25]. In our model, some degree of medial layer proliferation was observed in some arteries, which is consistent with a direct action of rHuEPO on these cells (Fig. 6).

It is well established that rHuEPO therapy alters hemorheology [26], viewed by the Ht increase which causes blood hyperviscosity, thus increasing vascular resistance. Excessive erythrocytosis and/or polycythemia are conditions associated with an increase in viscosity and cellularity, which are responsible for a reduction in renal blood flow (RBF), renal oxygen delivery and, consequently, renal hypoxia [27]. The reduction in RBF is also responsible for increased vascular resistance, contributing to hypertension.

The increased expression of HIF2α in the rHuEPO200 and rHuEPO600 groups is consistent with the presence of renal hypoxia, due to blood hyperviscosity, which modulates tissue perfusion [28]. It was also reported that kidneys of hypoxic rats present hyperemia [29], which was also encountered in our study. However, considering the Hb levels observed in the rHuEPO200 and rHuEPO600 groups, the kidney tissue should be normoxic and, therefore, the HIF system would not be overexpressed.

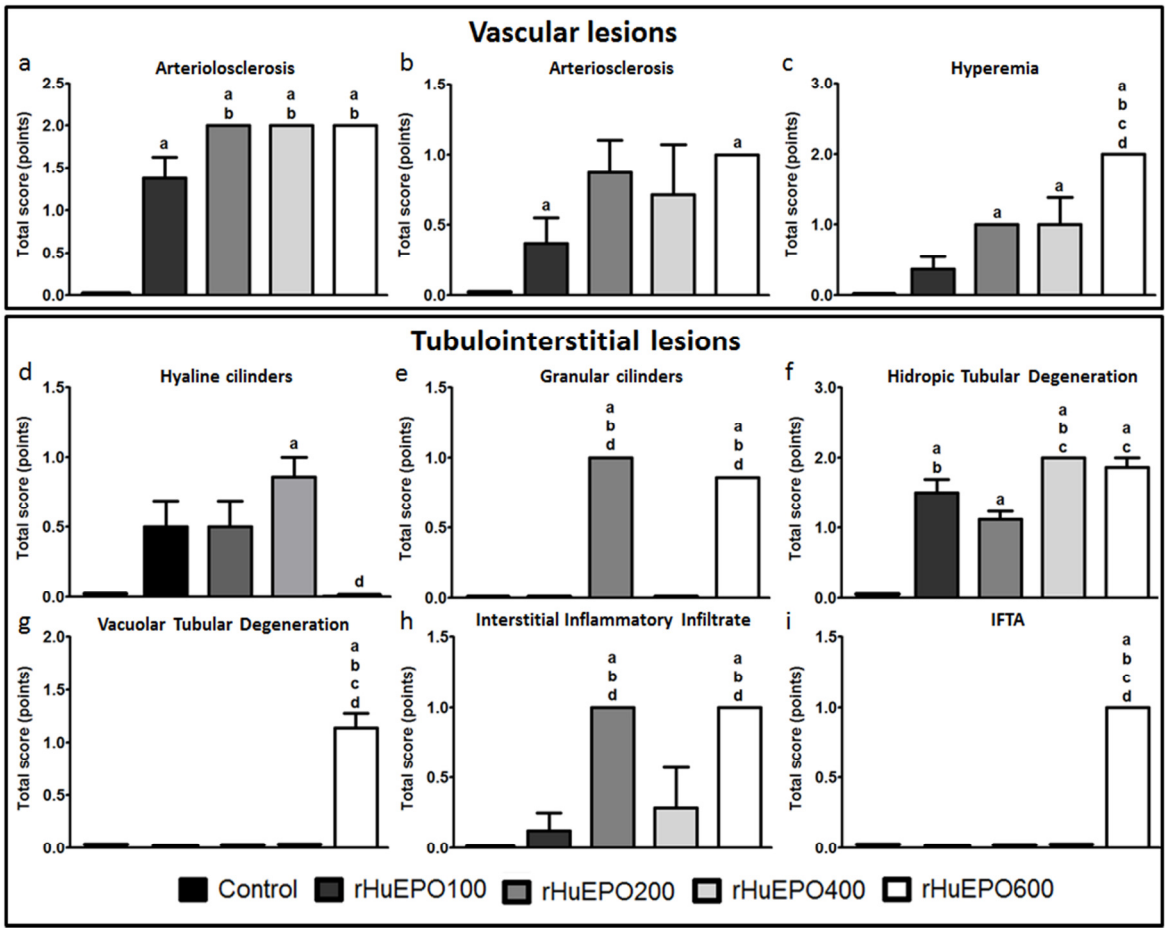

Fig. 3 – Total score for vascular and tubulointerstitial lesions at the end of protocol. Results are presented as Mean ± SEM: a) $p<0.05$ vs control group; b) $p<0.05$ vs rHuEPO100 group; c) $p<0.05$ vs rHuEPO200 group; d) $p<0.05$ vs rHuEPO400 group (Mann-Whitney Test).

As expected, some adaptive mechanisms to excessive erythrocytosis should be present in our model, in agreement with other studies. In fact, in a model overexpressing EPO, there was an adaption to excessive RBC mass by the regulation of blood viscosity, which is achieved by increasing the proportion of young RBC that are more flexible and, therefore, allow a reduced flow resistance [30]. Since Hb is the major intracellular protein of RBC, the MCHC reflects the protein content of the cell and, thus, the intracellular viscosity. An increase in the intracellular protein content leads to an increase in intracellular viscosity that will compromise RBC flexibility [31]. The presence of increased MCV and reticulocytes in the higher rHuEPO doses (rHuEPO400 and rHuEPO600), along with a reduction in MCHC, indicates the presence of a more young and flexible population of RBC, modulating the excessive erythrocytosis, which would favor and increase RBF, features that are not observed in the rHuEPO200 group. However, the organ capacity to respond to blood viscosity is only maintained if no injury was observed, which is not the case for the rHuEPO600 group, once the presence of interstitial inflammatory infiltrate, IFTA and granular cylinders is compatible with more pronounced degenerative processes, blunting the positive modulator effect of increased reticulocytes.

HIF system activation evoked an overexpression of kidney VEGFα mRNA and an increased serum VEGFα concentration, despite unchanged EPO mRNA expression in the renal tissue. The reduced

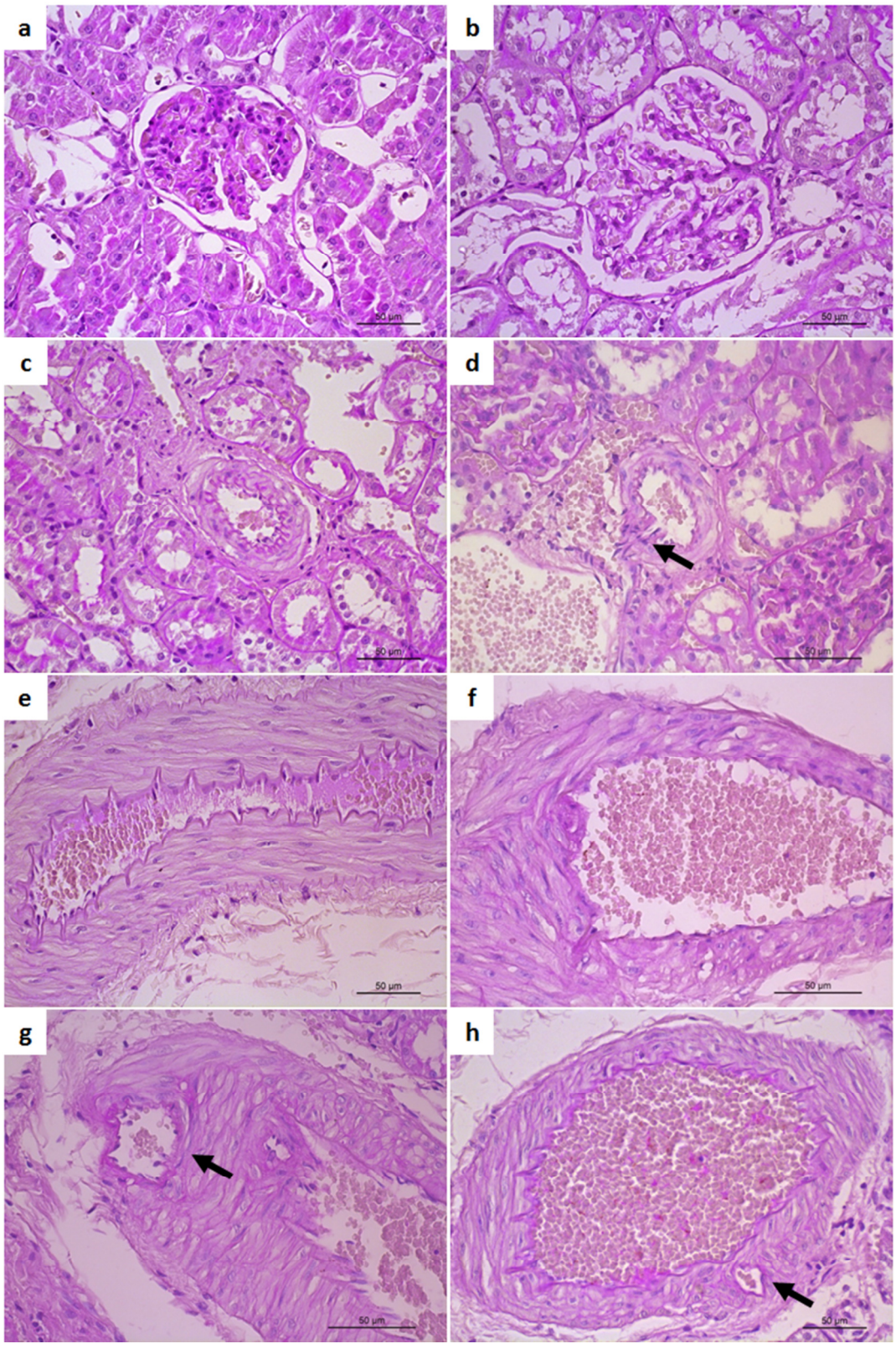

Fig. 4 – Representative images of glomerular and vascular lesions at the end of protocol (PAS staining, original magnification x400). a) normal glomerulus; b) glomerular hypertrophy; c) normal arteriole; d) arteriolosclerosis (grade 2) ; e) normal arteria; f) arteriosclerosis (grade 1); g) neovascularization and arteriosclerosis (grade 1); h) hyperemia (grade 2) and neovascularization.

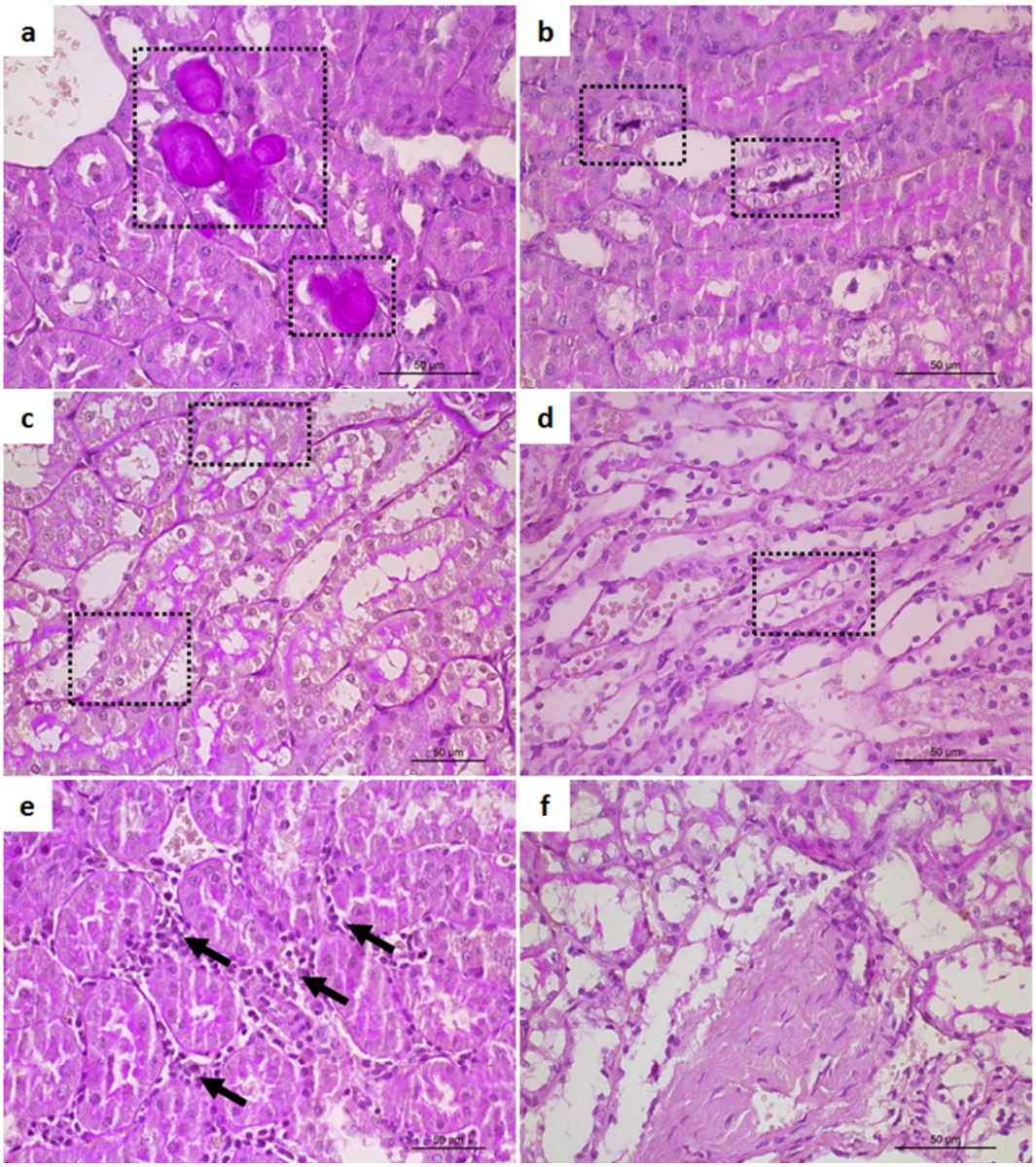

Fig. 5 – Representative images of tubulointerstitial lesions at the end of protocol (PAS staining, original magnification x400). a) hyaline cylinders (grade 1); b) granular cylinders (grade 1); c) hidropic degeneration (grade 1); d) vacuolar degeneration (grade 1); e) interstitial inflammatory infiltrate (grade 2); f) interstitial fibrosis and tubular atrophy (grade 1).

EPO mRNA expression could be due to the exogenous rHuEPO treatment, as elevated circulating levels of rHuEPO can supress endogenous EPO synthesis [15, 32, 33]. VEGF is responsible for angiogenesis in regions under hypoxia, increasing the blood supply [34], which is in agreement with the presence of neovascularization found in several animals treated with rHuEPO in our study.

In the context of hypertensive states, VEGF appears to be beneficial as it mediates vasodilation through the increase in eNOS activity that will improve NO availability. VEGF induces phosphorylation on ser1177 to increase eNOS activity, through the AKT or ERK signalling pathways [35]. Although the AKT pathway was increased in the rHuEPO200 and rHuEPO600 groups (Fig. 2E) (though not in the same extent as in the rHuEPO100 and rHuEPO400 groups), eNOS phosphorylation

was reduced (Fig. 2C). It is reported that hypoxia affects eNOS activity by promoting post-translation modifications or by decreasing eNOS mRNA transcription and reducing eNOS mRNA half-life [36], thus explaining our data. It is also important to refer that high levels of RBC and Hb are responsible from scavenging the NO released by endothelial cells, thus limiting its bioavailability [37]. Excessive erythrocytosis also alters the vascular system, by increasing vascular permeability and/or susceptibly to injury, which contributes to endothelial dysfunction [38]. The presence of a dysfunctional endothelium compromises vasodilation, justifying the BP raise due to increased peripheral vascular resistance (Fig. 6).

In the rHuEPO100 and rHuEPO400 groups, the increased eNOS phosphorylation seems to be modulated by the rHuEPO-evoked AKT signalling pathway, as the VEGF levels were not increased. EPO acts through the EPO receptor (EPOR)-activated STAT5 pathway to promote erythropoiesis; however, EPO is also responsible for non-hematopoietic effects, which are believed to occur due to the dimerization of EPOR and β Common Receptor (βCR) [39]. Recent findings suggest that this receptor may be responsible for the changes on eNOS activity mediated by EPO, which is in agreement with our study [40].

Previous studies performed in people leaving at high altitude have demonstrated that when RBF is reduced there is a simultaneous decline in renal plasma flow and an increase of filtration fraction, with unchanged GFR [27, 41], which is in agreement with our data. Considering that no change in GFR was observed in the higher dose group (rHuEPO600), the increased serum BUN levels may be due to non-renal causes, such as increased protein catabolism and/or hemoconcentration [42]. The kidney lesions observed in our model (glomerular hypertrophy and vascular and tubular damage) resembles those observed in individuals with excessive erythrocytosis and mild hypertension [27].

One major limitation of our study is the absence of kidney oxygenation measurements; indeed, it was reported that HIF system could be activated by an independent-oxygen pathway, through the AKT signalling pathway [43]; however the absence of HIF activation in the rHuEPO100 and rHuEPO400 groups, together with the other findings in the rHuEPO200 and rHuEPO600 groups, discard that hypothesis.

In combination, our findings suggest a direct effect of rHuEPO on renal vasculature, with increased VSMC proliferation, accompanied by increased Ht and blood viscosity, reduced eNOS expression, altogether contributing to the development of renal hypoxia and hypertension, culminating with the presence of vascular and tubulointerstitial lesions only with 3 weeks of rHuEPO treatment (Fig.6).

Despite the features of rHuEPO treatment observed in our study, other reports showed contrary observations. In fact, in rats with CKD induced by nephrectomy or with diabetic nephropathy, low doses of rHuEPO were able to correct anemia and ameliorate endothelial dysfunction by increasing eNOS activity, without affecting blood pressure [44-46]. The treatment with higher rHuEPO doses appears to present renoprotective and cardio-protective effects in animal models [47, 48] and in clinical studies [7, 49]. These controversial results should take into account the presence or absence of an underlying pathological condition, such as CKD. The use of rHuEPO to correct anemia presents benefits by reducing hypoxia and inflammation, which may restore the restore eNOS activity.

6. Conclusions

Our study showed that rHuEPO-induced hypertension might involve indirect (hematological) and direct (renal) effects which varies according to the

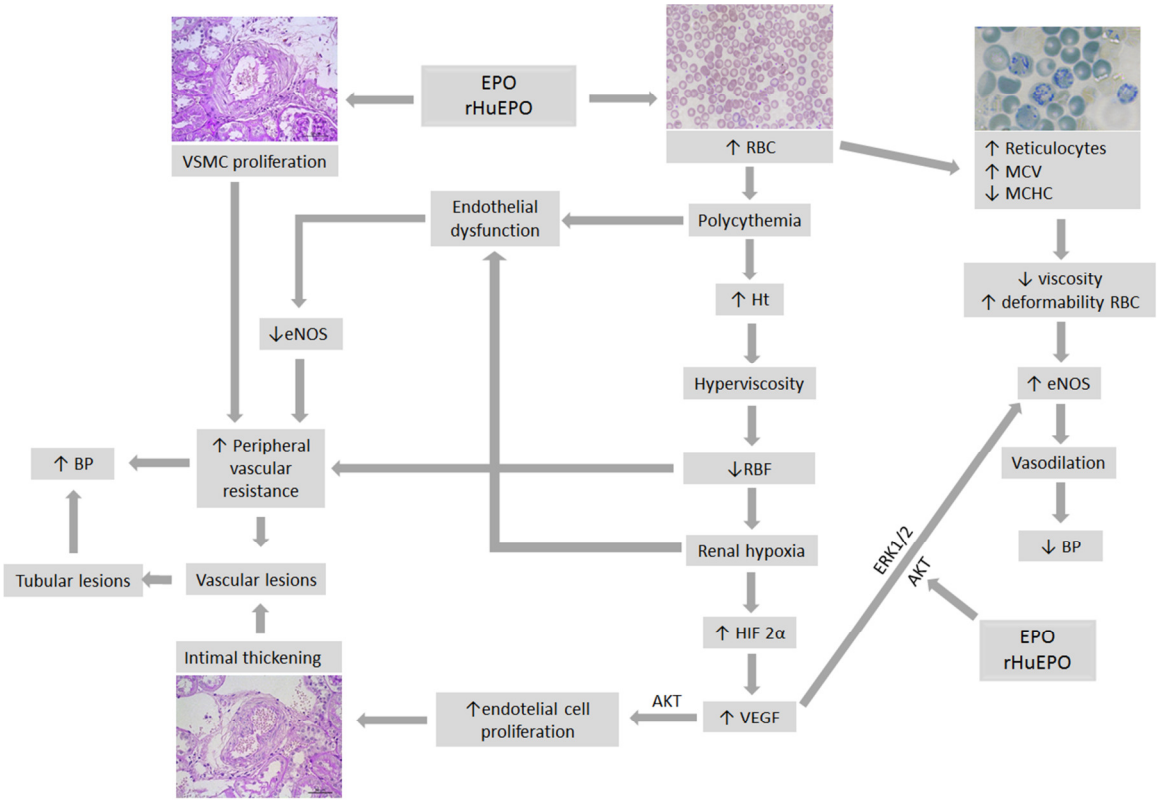

Fig. 6 – Schematic diagram of mechanisms involved in rHuEPO-induced hypertension. BP – blood pressure; eNOS – endothelial nitric oxide synthase; EPO – erythropoietin; Ht – hematocrit; HIF2a – hypoxia inducible factor 2 alpha; MCV – mean cell volume; MCHC – mean cell haemoglobin concentration; rHuEPO – recombinant human EPO; RBC – Red blood cells; RBF – renal blood flow; VEGF - vascular endothelial growth factor.

dose used. Thus, rHuEPO therapy should be used rationally and under adequate surveillance, as hypertension develops even with lower doses. Especial caution with higher doses should be taken, as rHuEPO-induced hypertension leads to early renal damage without alterations in traditional markers of renal function, thus masking the serious adverse effects and risks.

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Conflicts of interest

The authors report no conflict of interest.

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Paper V

Recombinant human erythropoietin-induced erythropoiesis regulates hepcidin expression over iron status in the rat

Sandra Ribeiro, Patrícia Garrido, João Fernandes, Susana Rocha, Petronila Rocha-Pereira, Elísio Costa, Luís Belo, Flávio Reis and Alice Santos-Silva

Recombinant human erythropoietin-induced erythropoiesis regulates hepcidin expression over iron status in the rat

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ABSTRACT

Erythropoiesis and iron metabolism are closely linked as iron is an essential player in hemoglobin synthesis and hepcidin plays a central role in iron metabolism. The control of hepcidin depends on several factors; however, the crosstalk between them is poorly clarified for different physiological and pathological conditions. Our aim was to study the impact of increasing recombinant human erythropoietin (rHuEPO) doses on erythropoiesis, iron metabolism and hepcidin, using a rat model.

Male Wistar rats, 12 weeks old, were divided in 5 groups: control (vehicle) and rHuEPO-treated groups (100, 200, 400 and 600 IU/kg body weight/week), 3 times per week, during 3 weeks. Hematological and iron data were evaluated, as well as, the expression of several genes involved in iron metabolism. Liver hepcidin protein was evaluated by Western Blot.

The rHuEPO treatment induced an increase in erythrocyte count, hemoglobin concentration and hematocrit in a dose dependent manner; the highest dose induced extramedullary erythropoiesis in the spleen. Transferrin saturation (TSAT) increased in the rHuEPO200, rHuEPO400 and rHuEPO600 groups. Tf receptor 2 (TfR2), hemojuvelin (HJV) and bone morphogenetic protein 6 (BMP6) mRNA levels were up-regulated in rHuEPO200 group. Matriptase-2 was down-regulated in rHuEPO200 group, and up-regulated in the other rHuEPO-treated groups. Hepcidin synthesis was increased in rHuEPO200 group, and repressed in the rHuEPO400 and rHuEPO600 groups.

Our study showed that when a high erythropoietic stimulus occurs, independent of tissue oxygenation, the hepcidin synthesis is mainly regulated by TSAT, through the TfR2/HJV/BMP6 pathway; however, when the erythropoiesis rate reaches a specific threshold, the extramedullary hematopoiesis is also triggered, and the control of hepcidin synthesis is switched to matriptase-2 that blunts the TfR2/HJV/BMP6 complex and, thus, inhibits hepcidin synthesis.

Keywords: erythropoiesis, hepcidin, iron, matriptase-2, transferrin saturation.

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1. Introduction

Erythropoiesis and iron metabolism are deeply linked, as iron is needed for hemoglobin (Hb) synthesis. An erythropoietic stimuli increases iron demand into the bone marrow. Body iron is acquired through the diet and is mostly contained in Hb of circulating red blood cells (RBC) [1]. Heme-iron is absorbed through a receptor on the apical membrane of the duodenal enterocyte, releasing iron after digestion. The non-heme iron is absorbed in the enterocyte through the divalent metal iron transporter 1 (DMT1) and released into the circulation by the iron exporter ferroportin (FPN), present on the basolateral surface of the enterocytes, and also present in macrophages and hepatocytes [1]. Iron is transported by transferrin (Tf) to the locals where it is needed for heme synthesis or to be stored. Iron levels are physiologically controlled by the balance between iron influx and iron demands, being hepcidin the major regulator. Hepcidin is a liver peptide that binds to FPN, inducing its internalization and degradation [2]; thus, hepcidin regulates iron absorption and mobilization to avoid iron deficiency or overload. Several factors can regulate hepcidin synthesis, such as iron stores, inflammation, erythropoiesis and hypoxia [3]. High concentrations of diferric Tf and inflammation stimulate hepcidin synthesis, whereas increased erythropoiesis and hypoxia inhibit hepcidin synthesis; however, the crosstalk between these factors is not well established.

Erythropoiesis-stimulating agents (ESA) are mainly used to treat anemia in chronic kidney disease patients (CKD) and in patients receiving chemotherapy [4]. The discovery of non-hematopoietic actions for ESA [5] opened the possibility of their use in the treatment of other pathologies [6-8]; however, to achieve those effects, higher doses of ESA are usually needed [9]. A successful treatment with ESA increases erythropoiesis and iron demand into the bone marrow, leading to an increase in iron absorption and mobilization. The interaction between erythropoiesis, iron absorption and iron stores, namely which of them prevails in the regulation of hepcidin synthesis, is not fully elucidated. Although, some studies showed that stimulated erythropoiesis, even in the presence of iron overload, inhibits hepcidin synthesis [10,11], others reported a dominance of liver iron stores over erythropoiesis [12].

In this study we aimed to study the effect of recombinant human erythropoietin (rHuEPO) therapy on erythropoiesis and on iron metabolism, and the effect of each factor on hepcidin regulation, using a rat model.

2. Materials and methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab. Inc., Chatillon-sur-Chalaronne, France), with 320-350 g of body weight (BW), were maintained in ventilated cages, in an air conditioned room, subjected to 12 h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. All animals received human care and animal experiments were conducted according to the European Communities Council Directives on Animal Care. The experiments were approved by the Portuguese Foundation for Science and Technology and the Local Ethics Committee (ORBEA: Organ Responsible for Animal Welfare) of the Faculty of Medicine from the University of Coimbra.

The rats were randomly divided in five groups (7-8 rats each group) receiving a subcutaneous (sc) injection of saline solution (control group) or of rHuEpo (NeoRecormon®, Roche, Basel, Switzerland) 100, 200, 400 or 600 IU/kg BW/week (rHuEPO100, rHuEPO200, rHuEPO400 and rHuEPO600 groups, respectively), 3 times per week, during 3 weeks.

2.2. Sample collection

Blood samples were collected at baseline, and 1 and 3 weeks (end of protocol) after starting rHuEPO treatment, with the rats under anesthesia (intraperitoneal) with 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncuture, from the jugular vein, into Vacuette® tubes without anticoagulant (to obtain serum) or with K_3EDTA (to obtain plasma) for hematological and biochemical studies. Plasma and serum aliquots were immediately stored at -80ºC until assayed.

At the end of protocol, the rats under anesthesia were sacrificed by cervical dislocation; liver, spleen and duodenum were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighted. In order to isolate total RNA, 0.2 g of liver, spleen and duodenum samples, from each rat, were immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) upon collection and stored at 4°C for 24h; afterwards, samples were frozen at -20ºC. For western blot analysis organs were immediately frozen with liquid nitrogen and stored at -80ºC.

2.3. Hematological and iron metabolism studies

RBC count, Hb, hematocrit (Ht), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red cell distribution width (RDW) and platelet count were assessed in whole blood K_3EDTA, using an automated counter (HORIBA ABX, Amadora, Portugal). Reticulocytes were evaluated by microscopic counting on blood smears, after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA).

Reticulocyte production index (RPI) was calculated as previously described by Hillman and Finch [13][(reticulocyte %/maturation index)*(Ht/normal Ht)], where normal Ht was the mean value presented by the control group, and the maturation index (maturation time of circulating blood reticulocytes that increase with premature release of reticulocytes from the bone marrow) was 1 for all groups.

Serum iron and ferritin were analyzed through automatic methods and equipment (ROCHE Integra 400, Roche Diagnostics, Basel, Switzerland). Serum Tf levels were evaluated by rat specific ELISA kit (Transferrin Rat ELISA Kit, abcam, Cambridge, UK). Tf saturation (TSAT) was calculated using the formula (Iron µg/dL*100)/(Tf mg/dL*2).

2.4. Spleen histological analysis

Spleen samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 4μm thick sections were stained with hematoxylin and eosin for extramedullary erythropoiesis analysis. All samples were examined by light microscopy (Microscope Nikon Eclipse Ci) and images were captured using a digital microscope camera (Nikon DS-Ri2).

2.5. Gene expression analysis

Liver, spleen and duodenum RNA isolation and integrity control were performed as previously described by us [14]. One microgram of total RNA was reversely transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA), according to manufacturer instructions. One nanogram of cDNA was used for gene expression analysis with qPCR, using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs (Table 1). qPCR reactions are performed using the following conditions: enzyme activation at 95ºC for 40s; denaturation at 95ºC for 3s; annealing for 30s (for each pair primer temperature, refer to Table 1). Gene expression was normalized to actin beta

(Actb) and alpha tubulin (α-tubulin), and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. The expression of the following genes was analyzed in the liver: *bmp6* – BMP6*; hamp* – hepcidin*; hfe* – HFE; *hfe2* - HJV*; tfrc* – TfR1*; tfr2* – TfR2*; tmprss6* – matriptase-2; in the spleen we analyzed the gene expression of *epor* (EPO receptor), and in the duodenum the expression of *slc11a2 gene* (DMT1).

Table 1 - List of primer sequences and annealing temperatures

Gene	Primer sequences (5' → 3')	Annealing temperature
actb	F:TACAGCTTCACCACCACAGC R:AAGGAAGGCTGGAAGAG GC	57ºC
bmp6	F:GGTGGAGTACGACAAGGAGTT R:GTCACAACCCACAGATTGCTA	56ºC
epor	F:CCGGGATGGGCTTCAACTAC R:TCCAGTGGCACAAAACTCGAC	59ºC
hamp	F:GGCAGAAAGCAAGACTGATGAC R:ACAGGAATAAATAATGGGGCG	58ºC
hfe	F:TGGGCAAGATCACCTTGAATT R:GGATCCTGTGCTCTTCCCACT	58ºC
hfe2	F:TTCCAATCCTGCCTCTTTGAT R:GGAAAAGGTGCAAGTTCTCCAA	58ºC
slc11a2	F:ATAGCAGACGCCCCCATG R:AGGCCCGAAGTAACATCCAA	58ºC
tfrc	F:GGGAGCCATTGTCATACACC R:GTCGCAAAGCAGAGTCTTCC	58ºC
tfr2	F:AGCTGGGACGGAGGTGACTT R:TCCAGGCTCACGTACACAACAG	58ºC
tmprss6	F:AGAAGGTGGATGTGCAACTGATC R:CTTGCCCTTGCGATAACCA	59ºC
tuba	F:CACCCGTCTTCAGGGCTTCTTGGTTT R:CATTTCACCATCTGGTTGGCTGGCTC	59ºC

F: Forward; R: Reverse; *actb* - beta – actin; *bmp6* - bone morphogenetic protein 6; *epor* – erythropoietin receptor; *hamp* – hepcidin; *hfe* – hemochromatosis protein*; hfe2* - hemojuvelin; *slc11a2* - divalent metal transporter 1; *tfrc* – transferrin receptor 1; *tfr2* – transferrin receptor 2; *tmprss6* – matriptase-2; *tuba* – alpha-tubulin.

2.6. Protein analysis by Western Blot

Liver proteins were extracted using RIPA buffer (NaCl 150mM, Tris-HCl 50mM pH8, Triton X-100 1%, ethylene glycol tetraacetic acid 5mM, deoxycholic acid 0.5%, Sodium lauryl sulfate 0.1%) and ultra-sonication. After centrifugation, protein concentration of the supernatant was assayed using the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, IL, USA). Aliquots of the extract, containing 50 μg of protein, were separated by reducing SDS-PAGE (12 %) and transferred onto nitrocellulose membranes. The blots were blocked by using 7% non-fat milk in a solution of Tris-buffered salt with Tween-20. The nitrocellulose membranes were incubated, overnight at 4ºC, with goat anti-hepcidin 1:100 (sc-240553, Santa Cruz Biotechnoloy, Inc, Texas, USA), rabbit anti-SMAD1/5/9 1:500 (ab66737, abcam, Cambridge, UK) and rabbit anti-phospho SMAD1/5/9 1:500 (#13820, Cell Signaling Technology, Massachusetts, USA); afterwards, they were incubated with anti-goat (ab97120) or anti-rabbit (sc-2004) secondary antibody-conjugated with horseradish peroxide (abcam, Cambridge, UK and Santa Cruz Biotechnoloy, Inc, Texas, USA, respectively). Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL WesternBright, Advansta, California, USA). The analysis of the immunoblots was performed by densitometry (Bio1D++ version 99, Vilber Lourmat). To ensure even loading of the samples, all immunoblots were probed with rabbit anti-β-tubulin antibody 1:500 (sc-9104, SantaCruz Biotechnology, Texas, USA). The protein concentration in each sample was normalized for control group.

2.7. Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS) for Windows, version 22.0 (IBM, NY, USA). Results are presented as mean ± standard error mean (SEM). For comparison between groups Mann-Whitney test was performed. Statistical significance was accepted

at $p < 0.05$.

3. Results

3.1. Hematological data

The lowest rHuEPO dose group (rHuEPO100) showed an increase in Hb concentration and reticulocytes after 1 week of rHuEPO treatment, returning both parameters to values similar to those of the control group, at the end of protocol (Table 2). A significant increase in RDW was also found in the rHuEPO100 group.

The rHuEPO200, rHuEPO400 and rHuEPO600 groups showed a significant dose-dependent increase in RBC count, Hb concentration and Ht, more pronounced at the end of protocol (Table 2). After 1 week of rHuEPO treatment, we found a significant dose-dependent increase in reticulocytes and RPI, when compared to the control group; although the values remained significantly increased in the rHuEPO400 and rHuEPO600 groups, a reduction in reticulocytes and RPI from the 1st to the 3rd week was found. Throughout the study, a significant increase in MCV, and a reduction in MCHC were found in the rHuEPO200, rHuEPO400 and rHuEPO600 groups, together with an increase in RDW, when compared to the control group. At the end of the protocol, both rHuEPO400 and rHuEPO600 groups showed a significant reduction in platelet count, *versus* the other groups (Table 2). Serum EPO levels were significantly reduced in the rHuEPO100 group, when compared to the control and to the other rHuEPO-treated groups, in both follow-up times. At the end of protocol, the rHuEPO200 group presented significantly increased serum EPO levels, compared to the control and the other rHuEPO-treated groups.

3.2. Extramedullary erythropoiesis

No signs of liver extramedullary erythropoiesis were found; however, spleen extramedullary erythropoiesis was found in the rHuEPO600 group, as shown by the up-regulation of spleen EPOR mRNA levels (Fig. 1A). In addition, the histopathological analysis of spleen showed that the red pulp area was larger, compared to that observed in the other groups, and hematopoietic cells were observed only in this particular group (Fig. 1B-1D). Relative spleen weight (spleen/BW ratio) was also higher in the rHuEPO600 group, compared to the other groups (Spleen/BW ratio (g/Kg): control - 1.690±0.063; rHuEPO100 - 1.611±0.070; rHuEPO200 - 1.817±0.120; rHuEPO400 - 1.965±0.155 *versus* rHuEPO600 - 2.586±0.172, $p<0.05$).

3.3. Iron metabolism

Regarding serum iron metabolism, we found that only the rHuEPO100 group showed a significant reduction in serum iron when compared to the control and rHuEPO400 groups (Fig. 2A1). No significant changes were found in serum ferritin levels (Fig. 2A2). A reduction in serum Tf was observed in the rHuEPO100, rHuEPO200 and rHuEPO600 groups, when compared to the control group (Fig. 2A3). A significant increase in TSAT was encountered in the rHuEPO200, rHuEPO400 and rHuEPO600 groups, *versus* the control and the rHuEPO100 groups (Fig. 2A4).

A significant up-regulation in liver TfR1 mRNA levels was found in the rHuEPO600 group, when compared to the other groups (Fig. 2B1). Liver TfR2 mRNA levels were significantly overexpressed in both the rHuEPO200 and rHuEPO600 groups, *versus* the control and the rHuEPO400 groups (Fig. 2B2). No significant alterations were observed in HFE mRNA levels (Fig. 2B3). The rHuEPO200 group showed a significant up-regulation in HJV (Fig. 2B4), BMP6 (Fig. 2B5) and hepcidin (Fig. 2B6) mRNA levels, and a significant down-regulation of matriptase-2 (Fig. 2B7), compared to the other groups. A significant down-regulation of

Table 2 – Hematological data throughout the study (at baseline and after 1 and 3 weeks of treatment)

		Control	rHuEPO100	rHuEPO200	rHuEPO400	rHuEPO600
RBC (x 10^{12}/L)						
	0w	7.46±0.07	7.39±0.12	7.55±0.10	7.36±0.11	7.56±0.11
	1w	8.30±0.09	8.69±0.26	8.51±0.08	9.39±0.18***ac***	9.14±0.12***ac***
	3w	7.99±0.17	8.20±0.13	9.41±0.12***ab***	9.85±0.23***ab***	10.53±0.39***abc***
Hb (g/dL)						
	0w	14.44±0.18	14.44±0.17	14.31±0.21	14.29±0.17	14.56±0.18
	1w	14.96±0.18	15.99±0.16***a***	16.00±0.16***a***	17.09±0.19***abc***	17.60±0.25***abc***
	3w	14.47±0.19	14.74±0.30	17.06±0.32***ab***	18.14±0.40***ab***	19.60±0.60***abc***
Ht (%)						
	0w	42.42±0.52	41.17±0.47	43.14±0.66	41.26±0.61	42.49±0.53
	1w	43.69±0.73	45.37±1.30	48.74±0.71***a***	51.71±0.77***abc***	54.61±0.85***abcd***
	3w	43.57±0.85	44.89±1.19	53.21±1.16***ab***	57.37±1.20***abc***	66.56±1.03***abcd***
Ret (x10^{9}/L)						
	0w	228.01±13.21	151.84±13.25	243.03±17.41	215.25±29.06	256.93±16.96
	1w	305.32±13.32	566.05±30.32***a***	505.97±30.64***a***	959.73±44.64***abc***	1022.43±77.42***abc***
	3w	205.68±16.16	215.01±26.67	179.34±26.07	535.60±52.38***abc***	469.55±31.74***abc***
RPI						
	0w	2.93±0.13	2.04±0.19	2.90±0.07	2.86±0.17	3.12±0.23
	1w	3.65±0.13	6.81±0.43***a***	6.53±30.44***a***	12.05±0.58***abc***	14.67±0.91***abc***
	3w	1.80±0.27	2.43±0.26	2.30±0.35	7.07±0.63***abc***	6.40±0.50***abc***
MCV (fL)						
	0w	56.70±0.37	56.25±0.31	56.63±0.68	56.25±0.53	56.88±0.95
	1w	52.80±0.42	52.71±0.29	57.43±0.57***ab***	55.71±0.36***abc***	59.75±0.84***abcd***
	3w	54.50±0.48	54.88±0.35	57.14±0.59***ab***	58.29±0.71***ab***	59.86±1.03***abc***
MCH (pg)						
	0w	19.46±0.17	19.36±0.17	19.00±0.20	19.46±0.30	19.30±0.33
	1w	18.11±0.12	18.19±0.22	19.03±0.23	18.23±0.31	19.50±0.27
	3w	18.11±0.19	18.10±0.25	18.24±0.19	18.46±0.28	18.64±0.35
MCHC (g/dL)						
	0w	34.18±0.19	34.60±0.33	33.68±0.18	34.68±0.35	33.88±0.26
	1w	34.36±0.27	34.36±0.39	33.24±0.28***a***	33.04±0.29***ab***	32.24±0.14***abcd***
	3w	33.27±0.35	32.90±0.33	32.10±0.21***a***	31.63±0.11***abc***	31.14±0.36***ab***
RDW (%)						
	0w	12.22±0.11	12.74±0.16	12.39±0.18	12.61±0.13	12.30±0.21
	1w	13.50±0.23	15.20±0.11***a***	15.31±0.20***a***	17.41±0.20***abc***	16.93±0.25***abc***
	3w	13.34±0.06	14.21±0.23***a***	15.71±0.09***ab***	17.51±0.21***abc***	19.23±0.28***abc***
PLT (x 10^{9}/L)						
	0w	670.63±17.09	681.25±18.27	700.43±24.70	661.57±13.34	702.00±18.81
	1w	650.10±22.29	631.38±32.26	626.83±15.15	614.38±23.84	576.75±33.36
	3w	688.20±19.68	679.17±28.91	642.38±61.01	428.43±22.04***abc***	482.00±30.76***abc***
Serum EPO (mIU/mL)						
	0w	3.93±0.46	2.98±0.55	3.35±0.25	4.35±0.74	3.98±0.43
	1w	3.63±0.26	2.39±0.44***a***	5.11±0.86***b***	4.25±0.41***b***	3.40±0.19***b***
	3w	3.84±0.37	2.62±0.17***a***	6.61±0.58***ab***	3.47±0.39***bc***	3.38±0.26***bc***

Results are presented as mean ± SEM. ***a*** p<0.05 vs control group; ***b*** p<0.05 vs rHuEPO100 group; ***c*** p<0.05 vs rHuEPO200 group; ***d*** p<0.05 vs rHuEPO400 group (Mann-Whitney test). 0w –start of protocol; 1w – 1 week; 3w – 3 weeks ; EPO – erythropoietin; Hb – hemoglobin; Ht – hematocrit; MCH – mean cell hemoglobin; MCHC – mean cell hemoglobin concentration; MCV – mean cell volume; PLT – platelets; RBC – red blood cells; Ret – reticulocytes; RPI – reticulocyte production index.

BMP6 and hepcidin (Hamp) mRNA levels were observed in the rHuEPO400 and rHuEPO600 groups, accompanied by an up-regulation of matriptase-2. Duodenal DMT1 mRNA levels were up-regulated in the rHuEPO200, rHuEPO400 and rHuEPO600 groups, when compared to the control and the rHuEPO100 groups (Fig. 2B8).

While a significantly increased liver hepcidin protein expression was found in the rHuEPO200-treated rats, a reduced expression was observed in the rHuEPO400 and rHuEPO600 groups (Fig. 3A).

Liver pSMAD1/5/9:SMAD1/5/9 ratio protein was significantly increased in the rHuEPO200 group, but reduced in the other treated groups, com-

pared to the control group (Fig. 3B).

Discussion

The synthesis of hepcidin is known to be controlled by different factors; however the crosstalk between them in specific physiological and pathological conditions is poorly understood. rHuEPO is widely used due to its stimulating effect on erythropoiesis and to its pleiotropic actions, but to achieve the desired non-hematopoietic actions, high rHuEPO doses are needed. The stimulating effect on erythropoiesis is also illicitly used in sports doping to increase RBC mass, improving sports performance. Our aim was to clarify the impact of a stimulated erythropoiesis triggered by increasing rHuEPO doses, in case of normal tissue oxygen levels.

We found that the administration of increasing rHuEPO doses to healthy rats induced erythropoiesis in a dose-dependent manner, as showed by the increase in RBC, Hb and Ht. The highest dose (rHuEPO600) was also able to induce spleen extramedullary erythropoiesis, and the analysis of the blood smears showed the presence of Howell-Jolly bodies in the RBC, compatible with an altered splenic function [15]. The increase in MCV and the reduction in MCHC, observed in the rHuEPO200, rHuEPO400 and rHuEPO600 groups, are the result of a higher number of reticulocytes caused by stimulated erythropoiesis, which are larger than mature RBC and with lower Hb concentration. The increased RDW confirmed the anisocytosis due to the increased reticulocytes. The reduced platelet count observed, at the end of the protocol, in the rHuEPO400 and rHuEPO600 groups, might be due to competition between erythroid and megakaryocytic cells that have common progenitors cells [16]. Another possible explanation is that the hemoreological disturbances, due to increased RBC concentration, lead to platelet sequestration in the spleen, reducing its circulating number.

The increasing rHuEPO stimulus promoted an increase in iron absorption and mobilization, as suggested by the increase in TSAT and duodenal DMT1 mRNA levels, in order to face the increased iron demands for stimulated erythropoiesis, maintaining serum iron and ferritin levels (Fig. 2). Tf is responsible for iron transport to its target cells, carrying each molecule two iron (Fe^{3+}) ions. Tf-bound Fe (Tf-Fe) can be uptaked by the cells through two types of Tf receptors (TfR), TfR1 and TfR2; TfR1 is expressed ubiquitously and presents higher affinity for Tf-Fe; increased TfR1 levels can be found in accelerated erythropoiesis to compensate the high rate of iron demand for RBC production; TfR2 is mainly expressed on hepatocytes and presents other iron regulatory functions [17]. In conditions of normal TSAT, a sensor for systemic iron availability, Tf-Fe and the hemochromatosis protein (HFE) compete for binding to TfR1; in conditions of increased TSAT, Tf-Fe displaces HFE from TfR1 that binds to TfR2, activating the recruitment of hemojuvelin (HJV), a co-receptor of bone morphogenetic protein 6 (BMP6) [18]. HJV can be regulated by matriptase-2, which promotes its cleavage producing a soluble form that blunts the action of BMP6 [19]. The linkage of HJV to BMP6 leads to the phosphorylation of SMAD1, SMAD5 and SMAD9, activating the recruitment of SMAD4, forming a complex that will translocate into the nucleus and activate the transcription of the hepcidin gene [20]. The increase in hepcidin synthesis will lead to a reduction in iron absorption and mobilization, in order to control iron body levels. However, TSAT is not the only regulator of hepcidin synthesis. Erythropoiesis acts as an inhibitor of hepcidin synthesis, in order to stimulate iron absorption and mobilization for an efficient Hb synthesis by the erythroid cells. Regardless of the progresses achieved on hepcidin regulation, during the last years, the exact mechanisms by which erythropoiesis inhibits hepcidin synthesis are not fully

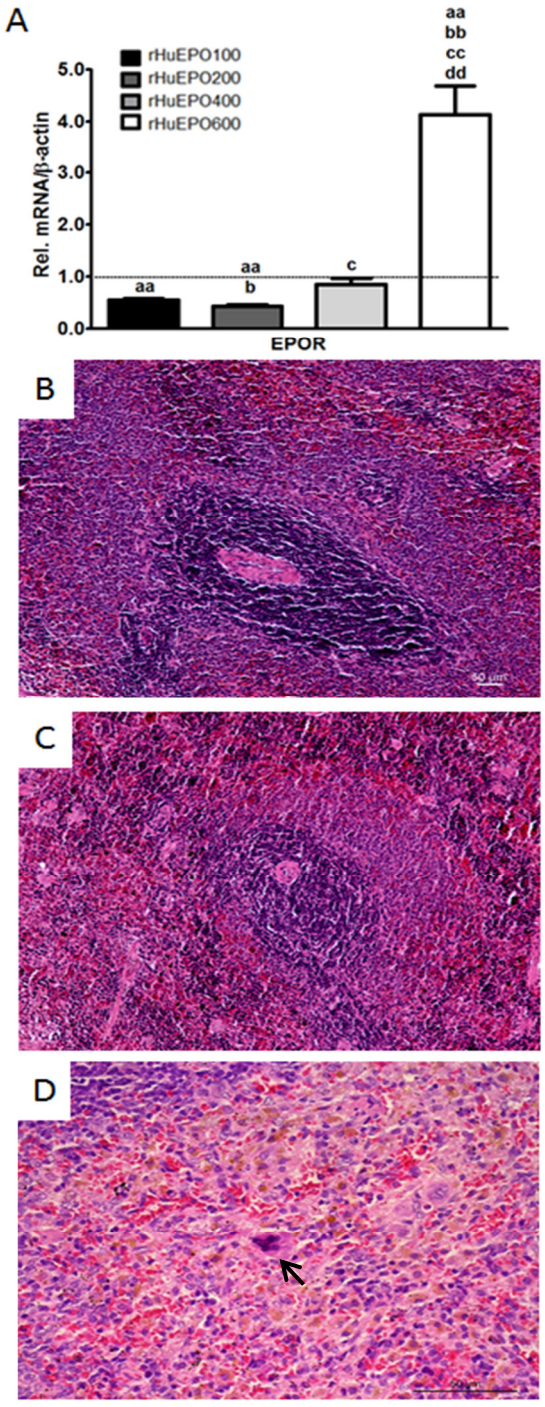

Fig. 1 – Extramedullary erythropoiesis in the spleen. A) Relative mRNA expression of erythropoietin receptor (EPOR) in spleen; B) Representative image of the white and red pulp area in the spleen of the control group (100x); C) Representative image of the enlarged red pulp area in the spleen of the rHuEPO600 group (100x); D) Representative image of erythroid cells and megakaryocyte (arrow) in the red pulp of the rHuEPO600 group spleen (400x).

understood. It was reported that EPO itself can be an inhibitor factor [21], but recent studies showed that in conditions of high EPO levels, without erythropoiesis, no inhibition of hepcidin synthesis occurs [22,23]. It appears that erythropoiesis inhibits hepcidin synthesis through some factors produced by the hematopoietic cells in the bone marrow, such as the growth differentiation factor 15 [24], the twisted gastrulation factor-1 [25] and, the erythroferrone [26,27].

In the rHuEPO200 group, the erythropoiesis stimuli triggered by rHuEPO seems to overwhelm the bone marrow response capacity, as suggested by the increase (two fold) in reticulocyte count and RPI at the 1st week that was followed by a decrease to basal values at the 3rd week of treatment. The significantly high serum EPO levels in the rHuEPO200 group (Table 2) and the up-regulation of liver EPO mRNA levels (data not shown) are consistent with a reduction in the bone marrow erythropoietic activity. Indeed, it was reported that serum EPO levels increases, in response to a reduction in erythroid progenitors in the bone marrow, independently of changes in tissue oxygenation [28]. Moreover, in line with this, the higher rHuEPO stimuli (rHuEPO400 and rHuEPO600) seem to trigger extramedullary erythropoiesis, already evident in the spleen, for the highest rHuEPO doses. In the rHuEPO200 group, we also found an up-regulation of liver TfR2, HJV and BMP6 levels, an activation of the SMAD1/5/9 pathway and a down-regulation of matriptase-2 that, altogether, contribute to increase hepcidin synthesis. It seems that in the dynamic balance, erythropoiesis-iron-hepcidin, the increased TSAT prevails over the inhibitory effect of erythropoiesis, increasing hepcidin synthesis to avoid iron overload. We found that while increased TSAT was a common feature in rHuEPO200, rHuEPO400 and rHuEPO600 groups, hepcidin synthesis was only increased in the rHuEPO200 group,

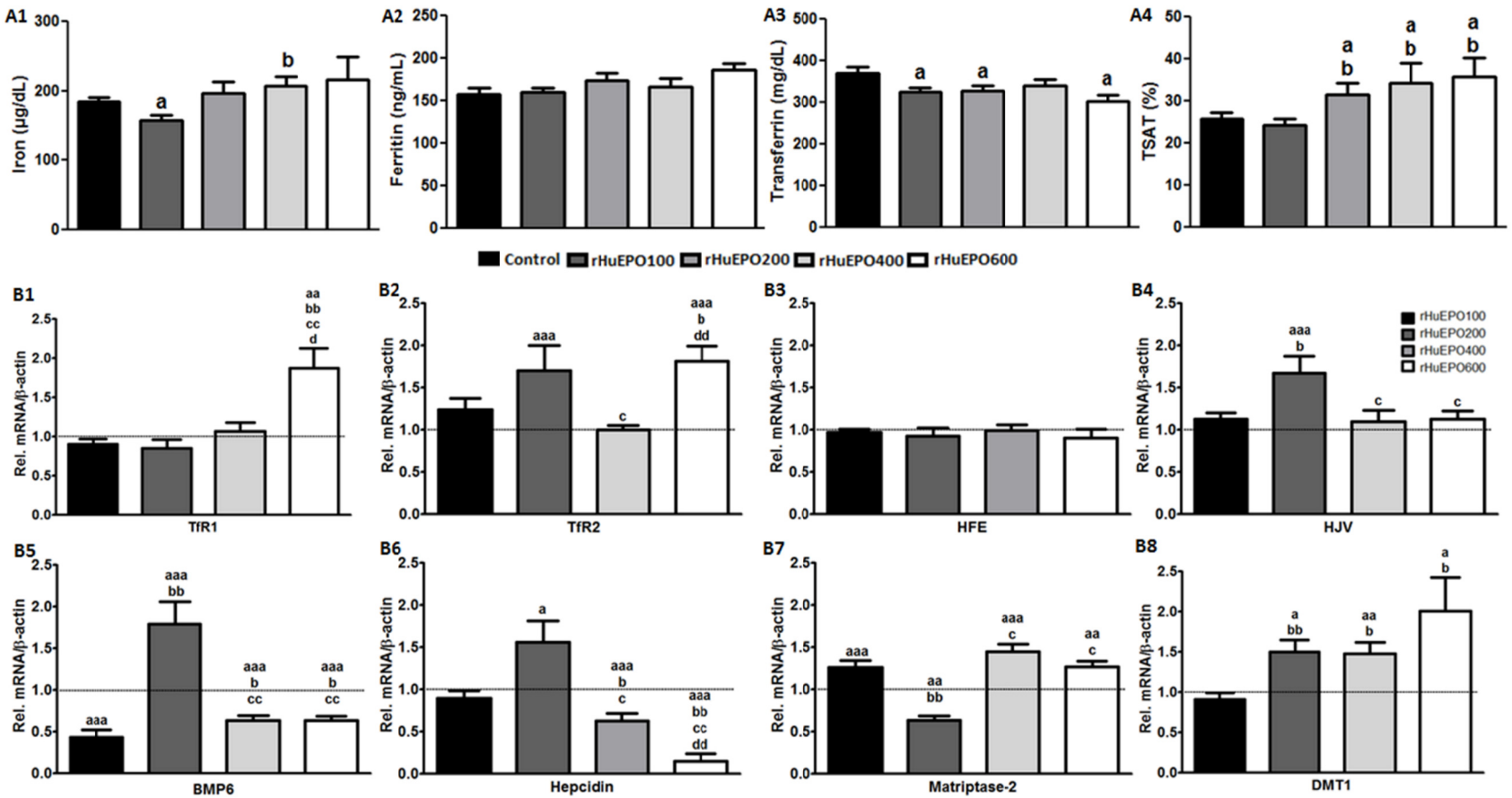

Fig. 2 – Iron data (A1-A4) and relative mRNA expression of liver (B1-B7) and duodenum (B8) genes involved in iron metabolism at the end of the protocol. Results are expressed as mean±SEM. a $p<0.05$, aa $p<0.01$, aaa $p<0.001$ vs Control group; b $p<0.05$, bb $p<0.01$ vs rHuEPO100 group; c $p<0.05$, cc $p<0.01$ vs rHuEPO200 group; d $p<0.05$, dd $p<0.01$ vs rHuEPO400 group (Mann-Whitney Test).

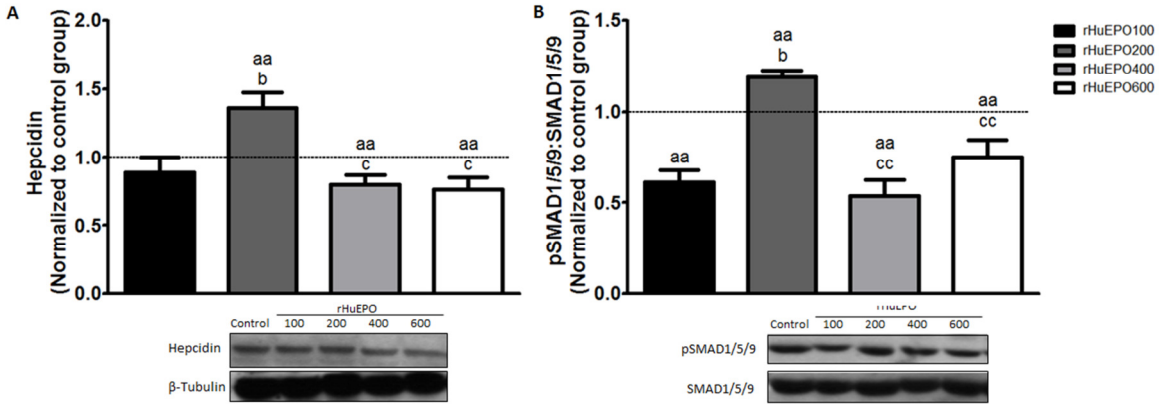

Fig. 3 – Evaluation of liver proteins by western blot and representative images of western blot for the different groups. A – Hepcidin; B – pSMAD1/5/9:SMAD1/5/9 ratio. Results are presented as mean ± SEM: aa $p<0.01$ vs Control group; b $p<0.05$ vs rHuEPO100 group; c $p<0.05$, cc $p<0.01$ vs rHuEPO200 group (Mann-Whitney test).

suggesting that other(s) factor(s) prevailed over TSAT in the modulation of hepcidin synthesis. The rHuEPO400 and rHuEPO600 groups showed a down-regulation in BPM6 and HJV mRNA levels, as compared to rHuEPO200 group, which translates in no activation of the SMAD1/5/9 pathway. In accordance, the rHuEPO400 and rHuEPO600 groups showed hepcidin mRNA levels and protein synthesis repressed. Although, TfR2 levels were not repressed, as they are regulated by TSAT, it appears that the increase in matriptase-2 is the main responsible for the inhibition of hepcidin transcription. In fact, matriptase-2 can be regulated directly by iron levels in order to prevent excessive hepcidin production [17]. We must also consider that the increase in blood viscosity, due to the increase in RBC, by inducing hemorheological disturbances may create a hypoxic environment in the microvasculature of the kidney and liver, also contributing to the switch in hepcidin synthesis. Moreover, given the high rate of erythropoiesis, showed by the high RPI and reticulocytes in the rHuEPO400 and rHuEPO600 groups, this could be another factor explaining the inhibition of hepcidin synthesis to face the high iron demands for such a high rate of normal and extramedullary erythropoiesis.

This dynamic balance between erythropoiesis and iron metabolism is frequently observed in patients with β-thalassemia, where the presence of increased erythropoiesis (but ineffective) results in inappropriate low hepcidin levels despite iron overload, showing the predominance of erythroid over iron as hepcidin regulator [27,29,30].

In conclusion, our study showed that when a high erythropoietic stimuli occurs, independent of tissue oxygenation, the hepcidin synthesis is mainly regulated by TSAT, through the TfR2/HJV/BMP6 complex that activates the SMAD1/5/9 pathway; however, when the erythropoiesis rate reaches a specific threshold, the extramedullary hematopoiesis is also triggered, and the control of hepcidin synthesis is switched to matriptase-2 that by inactivating HJV, blunts the TfR2/HJV/BMP6 complex and, thus, inhibits hepcidin synthesis.

Conflict of interest

The authors reported no conflicts of interest.

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DISCUSSION

General discussion

Anemia is a common complication in CKD patients that often develops early in the course of the pathology, being associated with the progression of the renal disease [22, 40, 41]. The first treatment available for anemia correction in CKD patients was RBC transfusion, but the introduction of rHuEPO in the late 80's revolutionized the treatment of anemia in CKD patients. The search for new and better formulations amplified the number of ESA available for anemia correction, but until today the treatment of anemia in CKD patients is still a challenge. Some of the CKD patients do not properly respond to ESA treatment [50] needing higher doses to overcome ESA resistance in order to achieve target Hb levels. Reported data showing an increased incidence of cardiovascular events and mortality in CKD patients under ESA therapy [243, 321], especially in hyporesponsive patients [250-254], raised concerns. In agreement, experts have reviewed, and updated in 2012, the guidelines for anemia management in CKD patients [48] and the use of more conservative ESA doses in clinical practice was recommended. However, the risks/benefits for high ESA doses are still poorly clarified, as well as the causes (and mechanisms) underlying the associated increased cardiovascular and mortality risk in CKD patients. Our experimental work aimed to contribute to clarify the linkage(s) of risk in the treatment of anemia with higher therapeutic doses of rHuEPO in CKD patients.

Animal models can be a valuable tool to study the pathophysiology of CKD anemia, the molecular mechanisms underlying the progression of renal disease and the effects of rHuEPO therapy beyond the correction of anemia, providing identification of putative new targets to improve therapeutic efficacy and reduce the high mortality observed in CKD patients. Previous studies conducted by our group showed that the remnant model of CKD induced by a ¾ nephrectomy presents a moderate stage of CRF, but without a sustained anemia [344]. Furthermore, we also showed that the 5/6 nephrectomy-induced CKD model was associated with the development of sustained CRF and anemia along the 9th week protocol [313]; however, the development of antibodies against rHuEPO after the 6th week of treatment [345] lead us to shorten, in the present study, the protocol time to 3 weeks of rHuEPO therapy.

In the work presented herein, after 3 weeks of nephrectomy, the rat model of CRF developed a moderate degree of renal failure, as showed by the 2 to 3 fold increase in serum creatinine and BUN, the remarkable increase in albumin excretion and the consistent reduction in GFR, which are markers of CKD used in clinical practice for the classification and stratification of the disease [1]. The development of renal insufficiency was associated with a high score of renal lesions (namely glomerular, tubulointerstitial and vascular lesions), hypertension, inflammation and anemia. The substantial surgical removal of nephrons induced a compensatory hypertrophy of the remnant kidney in the

CRF rats, with increased workload of the remaining nephrons that, by affecting other kidney structures, lead to the progression of the disease. The development of a moderate anemia in the CRF rats, following kidney mass reduction, is due to the reduction of renal EPO secretion, as showed by the reduced kidney protein expression of HIF-2α and EPO; however, serum EPO was increased, being mostly from hepatic origin. It has been reported that in both CKD patients and animal models of CKD, the serum EPO levels are within the normal range or even increased [62, 64-66, 313]. The serum EPO levels in CRF rats are, probably, not sufficient to overcome renal anemia; actually, anemic patients with normal renal function might increase serum EPO levels to 10 to 100 fold the normal value [67], depending on the disease severity [62, 63]. The kidney inflammatory features observed in the CRF rats (IL-6, IL-1β and NF-κB overexpression) may also contribute to reduce renal EPO production. It is known that patients with anemia can switch EPO production from kidney to liver [70]. Indeed, the increase in liver HIF-2α, EPO and EPOR, supports the contribution of the liver to serum EPO levels observed in CRF rats, as reported by others [346, 347] (**Paper I**).

The continuous hypoxic stimulation in the CRF rats may perpetuate the inflammatory response and the release of pro-fibrotic factors. Indeed, the sustained overexpression of TGF-β1 in CRF rats, known to increase the production and accumulation of ECM, thus promoting fibrosis [163], may explain the increased fibrosis and glomerulosclerosis and the increased expression of pro-inflammatory cytokines in CRF rats, further aggravating renal injury.

The use of rHuEPO in the CRF rats was able to correct the anemia in a dose-dependent manner. Comparing all the CRF groups under rHuEPO treatment (Table 12), we found that the lower doses tested (CRF100 and CRF200 groups), mimicking those usually used to correct anemia in CKD patients, were able to improve (but still present) glomerular and tubulointerstitial renal lesions, to reduce renal inflammatory features, but not systemic inflammation (CRP levels). The higher rHuEPO doses (CRF400 and CRF600), representing those used in the treatment of hyporesponsive CKD patients, markedly improved all renal lesions and inflammation (local and systemic). However, no differences in GFR were observed between the untreated group and those treated with rHuEPO. All the treated groups presented mild tubulointerstitial lesions that could explain the absence of renal function improvement. Actually, mild tubular lesions correlated significantly and negatively with GFR and positively with serum creatinine, in agreement with other studies [26]. Moreover, the histopathological analysis of the remnant kidney of rHuEPO-treated rats showed a dysfunctional tissue adjacent to the scar areas that may contribute to hamper GFR improvement (**Paper I**).

Hypertension is strictly linked with CKD, being either a cause or a consequence of the disease. Indeed, our CRF rats showed a hypertensive state, which was generally ameliorated by rHuEPO therapy; however, within treated groups rHuEPO presented a pro-hypertensive effect in a dose-dependent manner, which can blunt the beneficial effects on renal function (**Paper I**).

Several mechanisms have been proposed to explain the hypertension induced by ESA therapy [241]. In order to assess the effect of rHuEPO therapy *per se*, without the interference of CRF-induced changes, we treated normal rats with the same doses used in the CRF rats. We found that the hematological changes were rHuEPO dose-dependent, as occurred in the CRF rHuEPO-treated animals, leading to increased blood viscosity. An increase in blood pressure was observed in all treated animals that can result from the direct action of rHuEPO on the renal vessels [232, 233] and/or from the increased blood viscosity associated with erythrocytosis (**Paper IV**).

The increasing RBC, by increasing blood viscosity, cause an increment in vascular resistance; however, the observed increase in SBP cannot be only explained by the erythrocytosis, as the lower dose used was able to induce an increase in SBP without significant changes in RBC, suggesting a direct effect of rHuEPO on renal vessels, especially on VSMC, as previously reported by others [232, 233]. Indeed, we found a proliferation of the medial layer of the arteries and the presence of arteriolosclerosis and arteriosclerosis. The regulation of blood pressure appears to be mainly associated with eNOS activity, as the groups with higher blood pressure presented a reduction in eNOS activity. After 3 weeks of rHuEPO treatment, the groups with the highest blood pressure presented already arteriosclerosis and several tubulointerstitial renal lesions. Nevertheless, the markers used in this study to evaluate renal function and injury did not detect alterations in renal function (**Paper IV**).

These results are in accordance with data from a previous study conducted by our group [345], in which the treatment with rHuEPO during 9 weeks (with a dose of 200IU/Kg BW/week) was associated with an aggravation of advanced lesions. These worsening features were accompanied, among others, by a rise in blood pressure, regardless of similar hematological values (*versus* Sham group), suggesting that the higher renal damage was mainly due to rHuEPO direct effects on renal vessel and/or to rHuEPO-induced hypertension, rather than to increased Hb concentration and blood hyperviscosity.

Functional iron deficiency is a common feature observed in CKD patients, mainly in patients with resistance to ESA therapy [31, 90, 279], due to the increase in hepcidin synthesis stimulated by inflammatory mediators [266]. Usually, CKD patients under HD present high levels of inflammatory markers (CRP and IL-6) and disturbances in iron

metabolism (lower serum iron and transferrin, and increased TSAT and ferritin) [31]. In our study, despite the inflammatory state, no significant changes in serum iron markers were observed in untreated CRF rats. It is known that hepcidin transcription is regulated by several factors (inflammation, TSAT, liver iron, hypoxia and erythropoiesis) and the preponderance of each factor is still unclear for different pathological conditions. In our study, the hypoxia associated to anemia in the CRF group and the stimulated erythropoiesis with the lowest rHuEPO dose (CRF100), seem to prevail in the down-regulation of hepcidin mRNA levels. The higher rHuEPO doses (CRF200 and CRF400) induced a higher erythropoietic stimulus, increasing iron absorption to maintain iron availability to the higher erythropoiesis rate, thus causing hepcidin synthesis repression (Table 12). The highest rHuEPO dose (CRF600) endorsed an even higher erythropoietic stimulus and iron absorption that lead to liver iron accumulation, which induced liver hepcidin transcription, through the SMAD pathway [101], creating a scenario similar to functional iron deficiency (**Paper II**). Our data suggest that the rHuEPO stimulus trigger a first wave to achieve correction of the anemia/hypoxia (CRF100), by inhibiting hepcidin synthesis, favoring erythropoiesis through an increase in iron absorption. The increasing iron absorption seems to lead to increasing liver iron storage (CRF600) that is able to switch hepcidin production, triggering hepcidin transcription via BMP6/SMAD pathway. Thus, hepatic iron-induced hepcidin synthesis prevails over the other regulator factors. This increase in hepcidin is not due to inflammation (CRF600), as CRP levels were normal. As reported by others, it appears that in a moderate degree of renal insufficiency hepcidin is correlated with iron stores, but not with inflammatory markers [348]. Moreover, the increase in hepcidin in the CRF600 group is not due to reduced excretion, as GFR was similar to the other CRF groups.

The dynamic balance between the several factors regulating hepcidin production was also studied in healthy rats. Therefore, once CRF is not present is these rats, inflammation and hypoxia (induced by anemia) were excluded, becoming erythropoiesis and iron the major potential regulators. The treatment with rHuEPO in healthy rats stimulated erythropoiesis in a dose-dependent manner, as well as iron absorption (rHuEPO200, rHuEPO400 and rHuEPO600), shown by the increase in duodenal DMT1 transcription and TSAT. Contrasting with the results obtained in the CRF animals, higher rHuEPO doses were unable to induce an increased hepcidin synthesis; instead, we found a down-regulation of hepcidin transcription with the highest doses, despite the increased iron absorption, suggesting the supremacy of erythropoiesis over iron in the regulation of hepcidin in the absence of hypoxia, in order to maintain an adequate iron availability for Hb synthesis (**Paper V**). The reduction of erythropoietic activity in the rHuEPO200 group lead to an increase in liver hepcidin and, as occurred in CRF rats, the SMAD

pathway is the major regulator of hepcidin transcription; however, in this case (without CRF) no liver iron overload was observed, and the TfR2/HJV complex is responsible for inducing BMP6.

Table 12 – Effects of low and high doses of recombinant human erythropoietin therapy in chronic renal failure rats compared to untreated animals

Parameters	No rHuEPO treatment*	Low rHuEPO doses§		High rHuEPO doses§	
		100	200	400	600
Hematological data					
RBC	↓	↑	↑	↑	↑
Hb	↓	↑	↑	↑	↑
Ht	↓	↑	↑	↑	↑
Ret	↔	↔	↔	↔	↔
Serum EPO	↑	↓	↓	↓	↓
Biochemical data					
Serum CRP	↑	↔	↔	↓	↓
Serum creatinine	↑	↔	↔	↔	↔
CC	↓	↔	↔	↔	↔
GFR	↑	↔	↔	↔	↔
AE	↑	↓	↓	↔	↔
Blood pressure data					
SBP	↑	↓	↓	↓	↓
DBP	↑	↓	↓	↓	↓
MBP	↑	↓	↓	↓	↓
Iron metabolism data					
Iron	↔	↔	↔	↔	↓
Ferritin	↔	↔	↔	↑	↑
Tf	↔	↔	↓	↓	↓
TSAT	↔	↔	↑	↑	↑
Hepcidin	↔	↔	↓	↓	↑
Renal lesions/inflammatory markers					
Glomerular	↑	↓	↓	↓	↓
Tubulointerstitial	↑	↓	↓	↓	↓
Vascular	↑	↓	↓	↓	↓
TGF-β1	↑	↔	↓	↓	↓
NF-κB	↑	↓	↓	↓	↓
IL-1β	↑	↔	↓	↓	↓
IL-6	↑	↓	↓	↓	↓

* compared to Sham group; § compared to no rHuEPO treatment; ↑ - increased; ↓ - decreased; ↔ - unchanged.

Hyporesponsiveness to ESA therapy has been associated with several causes [255-258]. In previous studies conducted by our group, we found that ESA resistance in HD patients is mainly linked with inflammation and functional iron deficiency [31, 90].

Several studies reported that ESA hyporesponsiveness is associated with progression of renal disease and increased mortality in CKD patients [253, 254].

In our study, we found that a group of rats receiving the rHuEPO dose of 200IU/Kg BW/week (rHuEPO200NR), after an initial correction of anemia, presented a sudden reduction in the response to the treatment, developing anemia. As observed in the untreated CRF animals, high serum EPO levels were detected, mainly from hepatic origin, in order to compensate anemia. Serum CRP levels and renal inflammatory markers (NF-κB, IL-1β and IL-6) increased, contributing to repress bone marrow erythropoiesis and renal EPO synthesis. Despite of the inflammatory features, no disturbances in serum iron markers used in this study were observed and a down-regulation of hepcidin expression was found, following the development of hypoxia. The increased serum EPO levels and the exogenous rHuEPO may also contribute to further inhibit hepcidin synthesis [120] (**Paper III**).

Our data suggest that inflammation is an important determinant in hyporesponsiveness to rHuEPO therapy, blunting the response to both the endogenous (hepatic) EPO and to the rHuEPO. This hyporesponse causes the reappearance of anemia that contributes to worsening of renal fibrosis and to the progression of CKD, thus explaining the poorer outcome of non-responders CKD patients (**Paper III**).

Final remarks

In conclusion, our data give further insights into i) the pathophysiology of CKD associated complications, ii) the impact of rHuEPO therapy on renal lesions, inflammation, iron metabolism and blood pressure and, iii) the pathophysiology of hyporesponsiveness to rHuEPO therapy and of its impact on renal disease progression.

Finally, our data enable us to propose some answers to the starting question:

Anemia and high therapeutic doses of recombinant human erythropoietin in chronic kidney disease – a linkage of risk?

- A short-term treatment with high doses, used to overcome an episode of hyporesponse to rHuEPO, can present benefits by reducing inflammation, without worsening of renal lesions.

- The pro-hypertensive effect during a long-term treatment, particularly with higher rHuEPO doses, should be considered, and carefully managed in order to avoid a negative cardiorenal impact. This should also be considered in non-hypoxic conditions.

- The correction of anemia with high rHuEPO doses, by favoring erythropoiesis and iron absorption, can increase iron stores, inducing hepcidin synthesis that might contribute to hyporesponsiveness to rHuEPO therapy.

- Hyporesponsiveness to rHuEPO therapy associated with inflammation aggravates anemia, favoring hypoxia and renal fibrosis that will further enhance the inflammatory response, creating a vicious cycle that promotes renal disease progression.

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APPENDICES

Appendix I

rhEPO for the Treatment of Erythropoietin Resistant Anemia in Hemodialysis Patients – Risks and Benefits

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Chapter 16

rhEPO for the Treatment of Erythropoietin Resistant Anemia in Hemodialysis Patients – Risks and Benefits

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Additional information is available at the end of the chapter

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1. Introduction

Anemia is a common complication in hemodialysis (HD) patients, mainly due to the insufficient production of erythropoietin (EPO) by the failing kidneys [1]. Anemia itself can worsens cardiac function, cognitive function, exercise capacity and quality of life, and it has been independently associated with increased mortality and progression of renal disease [2, 3]. A successful management of anemia is, therefore, crucial, as it may improve clinical outcome. The introduction of recombinant human EPO (rhEPO) therapy to treat anemia of chronic kidney disease (CKD) patients reduced anemia, improving patients' quality of life [3]. There is, however, a marked variability in the response to this therapy and 5-10% of patients develop resistance to rhEPO therapy [4]. Resistance to rhEPO therapy has been associated to inflammation, oxidative stress and "functional" iron deficiency, as major causes.

EPO presents also an important protective role in other tissues, outside of the erythropoietic system. Actually, a biological response to EPO and the expression of EPO receptors, have been observed in many different cells, namely, in endothelial, neural and cardiac cells. However, HD patients requiring high rhEPO doses present an increased risk of death [5]. Recently, randomized controlled trials showed no benefit, or even increased risk of mortality and/or cardiovascular complications, in HD patients with hemoglobin (Hb) concentration higher than the target levels [6].

In this book chapter, a review of the etiological mechanisms associated with the development of EPO resistant anemia, in HD patients, will be performed. We also intend to review also the risk-benefits associated with high rhEPO doses used to achieve the target Hb levels.

2. Anemia of chronic kidney disease

CKD is a pathological condition that results from a gradual, permanent loss of kidney function over time, usually, months to years. CKD can result from primary diseases of the kidneys. However, diabetic nephropathy and hypertension have been considered as the main causes of CKD [1]. Anemia is a common complication of CKD that develops early in the course of the disease increasing its frequency with the decline of renal function. The incidence of anemia is less than 2 % in CKD stages 1 and 2, about 5% in CKD stage 3, 44% in CKD stage 4 and more than 70% in the end-stage renal disease (ESRD) [7]. This condition is associated with a decreased quality of life [3], increased hospitalization [2, 8], cardiovascular complications - angina, left ventricular hypertrophy (LVH) and chronic heart failure – and mortality [9-12].

The European Best Practice Guidelines for the management of anemia in patients with CKD recommends that a diagnosis of anemia in these patients should be considered when Hb concentration falls below 11.5 g/dL in women, 13.5 g/dL in adult men and 12.0 g/dL in men older than age 70 [13].

The anemia of these patients is, mainly, due to decreased kidney´s secretion of EPO. In CKD patients there is a failure in increasing the EPO levels in response to hypoxia, as occurs in others types of anemia. These patients present an EPO deficiency, rather than an absolute lack, as EPO remains detectable even in the most advanced stages of CKD [14]. However, other factors contribute to the anemia in these patients, as reduced red blood cell (RBC) life span, iron deficiency, uremic toxins, HD procedure, blood loss and inflammation.

3. Erythropoiesis-stimulating agents

The correction of anemia in CKD patients needs pharmaceutical intervention with erythropoiesis-stimulating agents (ESAs). An intravenous (i.v.) iron supplementation, as adjuvant therapy, should be administrated to prevent iron deficiency and minimize the dose of ESA needed to achieve the target-range of Hb levels [4, 13]. However, recently, some concerns about this treatment of the anemia were raised and questioned in several studies, namely, the need to define Hb targets, safety, benefits and costs of ESA treatments.

3.1. Pharmacology of erythropoiesis- stimulating agents

The introduction of ESAs revolutionized the treatment of anemia in CKD patients. After cloning of the EPO gene, the recombinant human technology allowed the production of ESAs that present the physiological role of EPO. Epoetin beta was the first ESA to be used. It was presented in 1987 [15] and approved by the Food and Drug Administration (FDA) in 1989. Since then, other ESAs appeared, with similar actions, differing in their half-life. Consequently, they were divided in “short-acting” and “long-acting” ESAs (Table 1). The frequency of administration and route of administration (usually, the intravenous (i.v.) administration is more convenient for HD patients) is, therefore, conditioned by their half-life.

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In humans, it seems that the rhEPO treatment increases Hb concentration, and, thus, arterial oxygen content, by increasing red cell volume and depressing plasma volume, probably through a mechanism involving the reduction of the renin–angiotensin–aldosterone axis activity [16].

The mechanisms for ESAs elimination are not well elucidated, and several hypotheses have been considered [17, 18]:

- ESAs are primarily cleared by a hepatic pathway;
- Clearance of ESAs occurs through the kidneys;
- ESAs may be cleared via EPO receptor-mediated endocytosis and subsequent intracellular degradation.

However, other mechanisms, not yet elucidated can be responsible for ESAs elimination.

ESA	Approval		Characteristics	Half-life	Frequency administration
	FDA	EMA			
Short-acting					
Epoetin beta		1989	Identical a.a. and carbohydrate composition to EPO	i.v. 4 - 12 h s.c. 12 – 28 h	3 times/week
Epoetin alpha	1989	1989	Identical a.a. and carbohydrate composition to EPO	i.v. ≈ 5h s.c. ≈ 24h	3 times/week
Epoetin zeta (biosimilar medicine)		2007	Identical a.a. and carbohydrate composition to EPO	i.v. ≈ 5h s.c. ≈ 24h	3 times/week
Epoetin theta (biosimilar medicine)		2009	Identical a.a. and carbohydrate composition to EPO	i.v.≈ 4h s.c. ≈ 34h	3 times/week
Long-acting					
Darbopoetin alpha	2001	2001	2 additional N-linked carbohydrate chains compared to EPO	i.v. 21 hours s.c. 73 hours	once/week
Methoxy polyethylene glycol-epoetin beta	2007	2007	continuous erythropoietin receptor activator	i.v. 134 hours s.c. 139 hours	once/month
Peginesatide	2012		PEGylated, homodimeric peptide with no sequence homology to rhEPO		once/month

Abbreviations: FDA – Food and Drug Administration; EMA – European Medicines Agency; a.a. – amino acid; i.v. – intravenous; s.c. – subcutaneous. rhEPO – recombinant human erythropoietin. Adapted from Food and Drug Administration (2012) [19], European Medecines Agency (2012) [20] and Green et al. (2012) [21].

Table 1. Erythropoiesis – stimulating agents.

3.2. Non-hematopoietic actions of erythropoietin and erythropoiesis- stimulating agents

ESAs are designed to treat anemia, but recent evidences points to other non-hematopoietic actions of EPO and ESAs [22]. Several pleiotropic effects have been attributed to EPO, such as cytoprotective, antiapoptotic, anti-inflammatory and angiogenic capacities.

The erythropoietic and non-erythropoietic effects of EPO appear to result from the existence of two different receptors with different affinities for EPO [23].

In erythroid cells, picomolar concentrations of EPO bind to the EPOR homodimers, whereas on other cells and tissues EPO binds to an heterodimer receptor, constituted by EPOR and CD131 (beta common receptor – βcR), and, high local EPO concentrations are needed to exert its action [23-25]. The EPO variants, including asialo-EPO, carbamylated EPO (CEPO) or carbamylated darbopoetin alpha (C-darbe), that present the protective effects of EPO in non-haematopoietic tissues, but no hematopoietic activity [26-28], suggested the presence of two types of receptors. EPOR are present in several cells and tissues, as brain (neurons, astrocytes, and microglia) [29, 30], kidney [31], female reproductive system [32], vascular endothelial cells [33], cardiomyocytes [34], lymphocytes and monocytes [35], among others.

Some of the non-hematopoietic effects of EPO are summarized:

- Cardioprotection: several studies showed that ESAs promote cardioprotection through the inhibition of cardiomyocyte apoptosis, reduction of inflammation and oxidative stress, and induction of angiogenesis [22-24, 34, 36].
- Anti-inflammatory properties: EPO and its derivates reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, and NO (nitric oxide) via inducible NO synthase (iNOS) through the inhibition of NF-κB pathway [23, 24, 37].
- Neuroprotection: EPO seems to be important for the neural development, as it stimulates the differentiation of neural progenitor cells [29], but it also promotes angiogenesis and reduces inflammation, oxidative stress and neuronal apoptosis in some conditions, as hypoxia-ischemia (HI), stroke and neurotoxicity of glutamate [22-24, 29].
- Angiogenesis: EPO increases the number of functionally active endothelial progenitor cells (EPCs), enhancing angiogenesis, and seems to be dependent on functional endothelial NO synthase (eNOS) [24, 38]. EPO plays an important role in uterine angiogenesis, through EPOR expressed by endometrial vascular endothelial cells [33].
- Immunomodulation: EPO may have effects on dendritic cells [potent antigen presenting cells (APCs) that possess the ability to stimulate naïve T cells], presenting effects in innate immunity [39].
- Renoprotection: several studies on acute kidney injury reported that a single dose of rHuEPO reduces kidney dysfunction through an antiapoptotic mechanism, and increased NO production, but only in intact vessels [31]. However, it appears that this renoprotection is achieved only with low doses of EPO, non-hematopoietic doses, as high EPO doses cause an increase in hematocrit that is accompanied with changes in hemorheology, activation of thrombocytes and increased platelet adhesion to injured endothelium [31].

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3.3. Benefits of erythropoiesis-stimulating agents

ESAs have beneficial effects by correcting anemia and their associated symptons (fatigue, dizziness, shortness of breath, among others), improving the quality of life of these patients [40-42]. ESAs also reduce the need for transfusions, thereby reducing transfusion reactions (immunological sensitization), transmission of infectious agents and iron overload [43].

The anemia of CKD is associated with cardiovascular complications, due to increasing blood pressure and LVH. Indeed, LVH is present in many patients with CKD, even in the earlier stages of the disease (75% of patients who start HD have LVH) and may lead to heart failure, cardiac arrhythmia or both, that are considered as major causes of cardiac-related deaths in this population [44, 45]. LVH is a physiological adaption that results from long-term increase of myocardial work, from high-pressure or volume overload, which can lead to major cardiac events. Volume overload can result from anemia, as hypoxia and the decreased blood viscosity contribute to decrease peripheral resistance, and from increased venous return, both of which increase cardiac output [44, 46]. LVH is also a risk factor for the development of uremic cardiomyopathy, which is defined as congestive heart failure due to a primary disorder of the heart muscle in uremic patients, and is characterized by profound systolic dysfunction and cardiac fibrosis; however, increased sympathetic activity in response to anemia also appears to be a factor for this condition [47, 48].

Several studies report the synergy between anemia and LVH and that the use of ESAs for anemia correction (Hb target of approximately 11 g/dL) is associated with an improvement in heart failure symptoms and with a reduction in LVH [45, 49].

The effects of ESAs on the progression of renal function are controversial. Some studies demonstrated that following ESA initiation renal function declines at a slower rate and delays the dialysis initiation in pre-dialysis patients [50-52], while other studies reported that ESAs do not significantly slow renal function decline [53, 54].

3.4. Risks associated with erythropoiesis-stimulating agents

As referred, ESAs have several benefits beyond the treatment of anemia; however, its administration seems to associate some risks. Cardiovascular and thromboembolic events have been described. Some of the protective effects of EPO and ESAs, as described above, occurs upon the activation of the heterodimeric EPOR; however, as the affinity of EPO for this receptor is low, higher doses of EPO are needed to reach these effects.

One of the most described effects of ESAs is hypertension. Several mechanisms can explain the rise in blood pressure (BP) mediated by ESAs. Renal anemia is a factor predisposing to increase BP, due to the increased sympathetic activity and impaired NO availability [55]. ESAs impair the balance between vasodilating and vasoconstrictor factors, since it induces the production of vasoconstrictors as endothelin-1 (ET-1), thromboxane (TXB2) and prostaglandin 2α (PGF2α), and reduces the production of the vasodilatory prostacyclin (PGI2) [56, 57]. Chronic treatment with ESAs appears to impair the vasodilatory capacity of endothelial NO, through an increase in the asymmetrical dimethylarginine (ADMA), an inhibitor of eNOS [57]. ESAs seem to induce hypersensitivity to angiotensin II, a recognized vasoconstrictor [56, 57].

An increase in noradrenaline concentration and hypersensitivity - a vasoactive substance - may contribute also to hypertension during ESA therapy [56, 57].

Treatment with ESAs is associated with an increase in the incidence of thrombotic events [58]. EPO has the capacity of stimulating thrombopoiesis, increasing platelet count; however, EPO also increases platelet reactivity (especially on the newly synthesized ones) promoting a prothrombic effect [59]. Some other hemostatic disturbances have been described, as an increased expression in E selectin, P selectin, von Willebrand factor and plasminogen activator inhibitor-1, which may favor bleeding episodes, and increase the risk of thrombosis and thromboembolism, as occlusion of the vascular access [57].

An uncommon but serious complication associated with ESAs administration is pure red blood cell aplasia, an immunogenic side effect that results from the production of antiEPO antibodies induced by ESAs administration [60-62]. Indeed, the method used to produce ESAs may not eliminate impurities or aggregated protein that may trigger the immune response in patients [62]. Immunoprecipitation assays have shown that antiEPO antibodies are directed against the protein moiety of the molecule [61].

ESAs are also indicated in the treatment of symptomatic anemia in adult cancer patients with non-myeloid malignancies receiving chemotherapy. However, some evidences point that these agents can accelerate tumor growth, but data are controversial. High doses of EPO can stimulate endothelial and vascular smooth muscle cell proliferation and promote angiogenesis. The antiapoptotic pleiotropic effect of EPO can also contribute to tumor progression [57, 63].

4. Resistance to erythropoieses-stimulating agents

Although the majority of CKD patients respond adequately to ESAs, 10% of these patients develops resistance to this therapy [4]. According to the European best practice guidelines for the management of anemia in patients with chronic renal failure [13] resistance to ESAs is defined as a failure to achieve target Hb levels (11– 12 g/dl) with doses lower than 300 IU/kg/ week of epoetin or 1.5 μg/kg/ week of darbopoietin-α. For the National Kidney Foundation Disease Outcomes Quality Initiative (NKF KDOQI) guidelines [4], hyporesponsiveness to ESAs therapy is defined by, at least, one of these situations: a significant increase in the ESA dose required to maintain a certain Hb level, a significant decrease in Hb level at a constant ESA dose or a failure to increase the Hb level to higher values than 11 g/dL, despite the administration of an ESA dose equivalent to epoetin higher than 500 IU/kg/week.

ESAs resistance is associated with poor outcome, increasing the risk of mortality [5, 64, 65]. Hyporesponsiveness to ESAs therapy can have many underlying causes. The most common causes are iron deficiency (absolute or functional), and inflammation.

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4.1. Iron deficiency

Iron-restricted erythropoiesis is frequent in CKD patients and is due to absolute or functional iron deficiency. The latter seems to be the most common cause of hyporesponsiveness to ESAs in HD patients [66, 67]. About 25-37% of CKD patients with anemia present with iron deficiency [66]. Iron therapy is recommended, and i.v. iron supplementation is more effective than oral supplementation in HD patients [67]. It is important to distinguish between absolute and functional iron deficiency. Indeed, there is a controversy about iron supplementation when transferring saturation is lower than 20% and ferritin is higher than 500ng/mL (functional deficiency) [67, 68]. In this situation, probably associated with an inflammatory response, an excess of iron can be potentially harmful to these patients.

4.2. Chronic blood loss

Blood loss is frequent in patients undergoing HD and could be a cause to an inadequate ESA response. This condition should always be suspected in several conditions, namely, in patients who need a higher dose of ESA to maintain a stable Hb concentration, in patients whose Hb concentration is falling, and in patients who fail to increase iron stores, even after i.v. iron supplementation [13].

4.3. Inflammation

The anemia of CKD is often referred as an inflammatory anemia. Indeed, inflammation is a common feature in CKD patients, mainly, in those under HD. Inflammation is recognized as one cause to hyporesponsiveness to ESA therapy, and several studies reported an association between high levels of inflammatory markers and ESA resistance in CKD patients [5, 69-72]. Usually, HD patients present with high levels of inflammatory markers, namely, IL-6, CRP, TNF-α, INF-γ, and with lower serum levels of albumin [69-71].

A week response to ESA also appears to be associated with enhanced T cell capacity to express IFN-γ, TNF-α, IL-10, and IL-13 [70, 73]. Costa et al. [71] also reported a significant rise in neutrophil count in non-responder patients. They also found positive correlations between CRP and elastase and between elastase and rhEPO doses, suggesting that elastase, a neutrophil protease released by degranulation, could be a good marker of resistance to rhEPO therapy in HD patients under hemodialysis. Inflammation contributes to anemia through several ways:

- suppression of erythropoiesis: **directly**, by the inhibitory effects of pro-inflammatory cytokines: IL-1β and TNF-α stimulate the growth of early progenitors BFU-E, but suppresses the growth of the later stages, inducing apoptosis in CFU-E [74]; **indirectly** as IL-1β and TNF-α stimulate the production of INF-γ [75], known to mediate erythropoiesis suppression.
- accelerated destruction of erythrocytes (as referred above in the uremic toxins section) by the reticulo-endothelial macrophages activated by the inflammatory state [76];
- reduction of EPO production: in hypoxic conditions, IL-1β and TNF-α increase the expression GATA and NF-κB, both inhibitory of the transcriptional factors of EPO gene [77];

- impaired iron availability for erythropoiesis: transferrin receptors in erythroid and non erythroid cells can be down-regulated by inflammatory cytokines reducing iron uptake [76]; they can also increase the expression of lactoferrin receptors and reduce the expression of ferroportin in macrophages, increasing the iron storage in these cells and reducing the iron availability [76, 78]; inflammation is responsible for the increase of hepcidin expression, a regulatory peptide in the iron cycling that reduces iron absorption and mobilization.

Recently, it was reported the existence of a soluble form of the EPOR (sEPOR) [79, 80]. Although this soluble receptor is able to bind to EPO, the role of these circulating sEPOR in humans remains largely unknown. sEPOR seems to be increased in patients receiving high ESA doses [79, 80], and the pro-inflammatory cytokines IL-6 and TNF-α can be responsible for this increment [79]. sEPOR could, therefore, be associated with ESA resistance through the inhibition of EPO effectiveness.

4.4. Decreased hepcidin excretion

In the last years hepcidin emerged as a key regulator of iron metabolism. Hepcidin is a peptide (25 aminoacids) produced, mainly, in hepatocytes, although other sites of production have been described, such as kidney [81], adipose tissue [82], brain [83] and heart [84, 85]. Hepcidin expression is regulated by the *HAMP* gene located in the long arm of chromosome 19 [86].

An increase of hepcidin levels leads to a decrease in iron absorption (hepcidin inhibits DMT1 transcription [87] or promotes an ubiquitin-dependent proteasome degradation of DMT1 [88]) and an inhibition of iron release from its storages (macrophages and hepatocytes) as hepcidin binds to ferroportin (the only known iron exporter in the cells) promoting its internalization and degradation in lysosomes (Fig. 1) [89, 90].

Hepcidin is increased in HD patients [91, 92], and it is regulated by inflammation [93] and linked to ESA resistance. Hepcidin correlates with IL-6, the cytokine that stimulate its production [94, 95], and with ferritin reflecting high inflammation and high levels of iron stores [96]. Some authors point that hepcidin could be a marker of functional iron deficiency [86] and that ESA therapy can decrease hepcidin levels [72, 96].

The kidney appears to play a role in the excretion of hepcidin, as this peptide is found in urine [97]. Hepcidin levels are increased in HD patients, and its levels appears to be reduced after HD procedure, supporting the role of kidneys in the excretion of this peptide [91, 92].

4.5. Secondary hyperparathyroidism

The parathyroid hormone (PTH) is considered by EUTox Work Group [98] as a middle molecule uremic toxin with some biological effects. Secondary hyperparathyroidism is a condition resulting from the deregulation of calcium and phosphorus homeostasis in the kidney. It seems that PTH could be a marker of hyporesponsiveness to ESAs in dialysis patients [99, 100].

Several mechanisms have been proposed as interference with RBC production as PTH causes bone marrow fibrosis, has an inhibitory effect on BFU-E and interferes with EPO endogenous

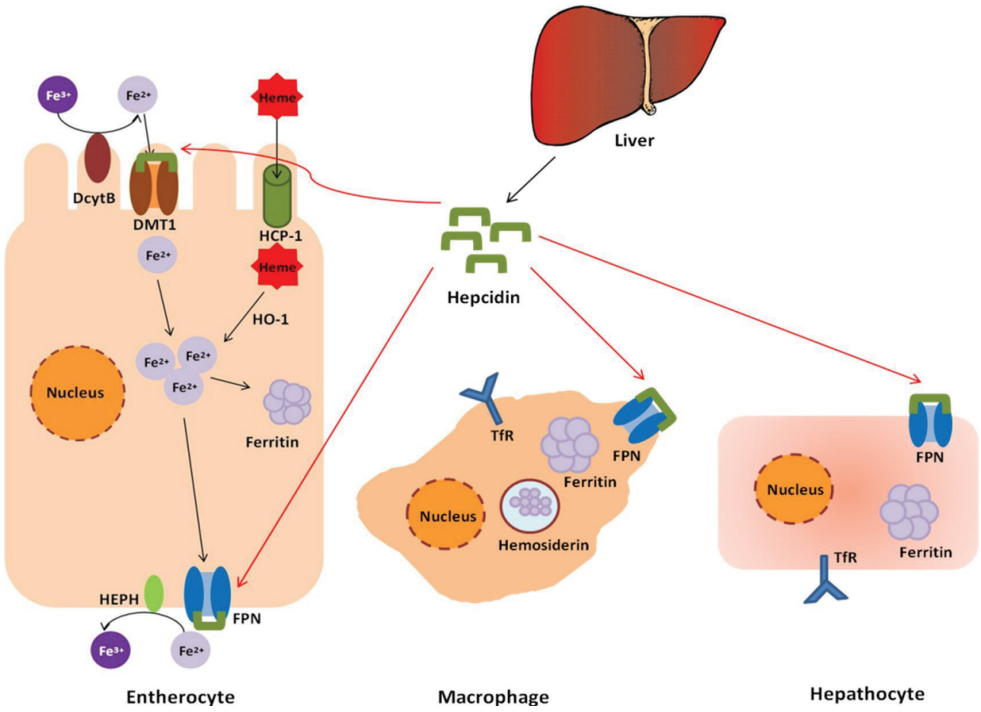

Figure 1. Iron metabolism and hepcidin. The iron is present in the diet as either heme iron (Fe^{2+}) or nonheme iron (Fe^{3+}). Nonheme iron must first be reduced to Fe^{2+}, by duodenal cytochrome B (DcytB), before it can be transported by the divalent metal iron transporter 1 (DMT1). Once inside the enterocyte, the newly absorbed iron enters the intracellular iron pool. If the iron is not required by the body, it is loaded onto the iron storage protein ferritin. Iron required by the body is transferred across the basolateral membrane by ferroportin (FPN). The export of iron also requires the ferroxidase hephaestin (HEPH). Heme carrier protein (HCP1) can transport heme; the enzyme heme-oxigenase 1 (HO-1) is required for releasing iron from heme. Hepcidin expression in the liver inhibits iron absorption from the diet and the release of iron from its storage.

production [99, 101-103]; interference with RBC survival as PTH increases osmotic fragility of erythrocytes [102, 103].

4.6. Aluminium toxicity

Although the recent progresses in the dialysis procedures, some patients present high levels of aluminium (Al) [104]. Usually, high levels of Al cause a microcytic, hypochromic or normochromic anemia that is hyporesponsive to ESA therapy, as it interferes with the enzymes necessary for the heme synthesis [67, 105]. The sources for the increase in plasma Al levels seems to be the water used for dialysis [105], medications given i.v. [104] and infections [106].

4.7. Vitamin deficiencies (e.g. folate or vitamin B12 deficiency)

The deficiency of folate or vitamine B12 is not very common in dialysis patients, but as these nutrients are water soluble and can be easily loss during dialysis, they can become a cause of ESA resistance, especially in patients with malnutrition. The supplementation of these nutrients seems to overcome ESA hyporesponsiveness [66, 67].

4.8. Malnutrition

Low body mass index (BMI) and low levels of cholesterol are related to poor outcomes in dialysis patients, increasing the risk of mortality [107]. This phenomenon, called as "reverse epidemiology", is based on the malnutrition-inflammatory complex [108]. These patients present a decreased nutritional reserve, reducing its capacity to overcome inflammation; they also present a reduced protein-calorie intake, chronic acidosis and failure of vascular access [108]. A diminished nutritional status and the enhancement of inflammation could be responsible for the requirement of higher EPO doses [69, 108].

4.9. Inadequate dialysis

Intensity or adequacy of dialysis (measured by Kt/V) is a factor that can modulate the response to ESA therapy. Inadequate dialysis is associated with the need for higher ESA doses. Some studies showed that convective treatments present benefits in ESA response, as compared with other treatments [109]. High flux HD (HF-HD) and online hemodialfiltration (OL-HDF) improve the response to ESAs, as compared to low flux HD (LF-HD), probably due to a better removal of middle and large molecules that impair erythropoiesis [67, 92, 109]. However, some studies failed to reach to these conclusions [110].

4.10. Angiotensin-converting enzyme inhibitors and angiotensin receptors blockers

These drugs, used for hypertension control, can be associated with ESA hyporesponsiveness due to its effects on angiotensin II. They can act through several mechanisms, not well understood, including inhibition of angiotensin-induced EPO release and increased plasma levels of N-acetyl-serylaspartyl-lysil-proline that impairs the recruitment of pluripotent hemopoietic stem cells [66, 67].

4.11. Testosterone deficiency

It appears that low testosterone levels may contribute to anemia in men with CKD and to ESA resistance. Testosterone stimulates erythropoiesis through the production of hematopoietic growth factors and possible improvement of iron bioavailability [111, 112].

5. Controversies in the treatment of anemia in chronic kidney disease

Since the introduction of ESAs therapy a demand exists to define the better Hb target associated with lower CV risks. Indeed, recent studies reported increased CV risk and death in patients

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treated with high doses of EPO to achieve higher Hb levels, and this led to the controversy of what is the cause of these increased risk: higher doses of EPO or higher levels of Hb?

5.1. Clinical trials

The correction of anemia to higher target Hb levels with ESAs in CKD or ESRD patients merits attention, as it may be associated with increased risk of death or of CV events, namely, stroke, hypertension, and vascular access thrombosis [6].

Only four studies assessed properly the effect of higher Hb levels on the increased risk of CV events and/or death.

5.1.1. Normal hematocrit trial (NHT) [113]

This study included patients under HD with congestive heart failure or ischemic heart disease. They were randomized to one of two groups to receive epoetin alpha, aiming to achieve and maintain a target hematocrit (Ht) of 42% or 30%. Primary end points were the length of time to death or for the first nonfatal myocardial infarction (MI). The study was interrupted due to the increased number of deaths observed in the high-Ht group and that were nearing the boundary of statistical significance. An increased rate of incidence of vascular access thrombosis was also reported in the high-Ht group. The study failed to reach statistical difference between the two groups, however, it was concluded that a target Ht of 42% is not recommended in HD patients.

5.1.2. Cardiovascular risk reduction by early anemia treatment with epoetin beta (CREATE) [53]

This study included pre-dialysis patients in stage 3 or 4 with mild-to-moderate anemia. They were randomly assigned to normalization of Hb values (13.0-15.0g/dL) or to a partial correction of anemia (10.5-11.5 g/dL), in order to investigate the effect of Hb correction on complications from CV causes. The primary endpoint was the time for the first CV event. Secondary objectives included the investigation of the effects of these treatments on the left ventricular mass index, the progression of CKD, and the quality of life. They did not find a significant difference in the risk for a first CV event between the two groups. However, this study reported a higher incidence of hypertension and headaches, and a higher risk for starting dialysis in the group aiming normalization of Hb values. But they also reported significant benefits on the quality of life for the patients with higher Hb targets.

In conclusion, they found that in pre-dialysis patients with mild-to-moderate anemia, the normalization of Hb levels to 13.0 to 15.0 g/dL did not reduce CV events.

5.1.3. Correction of hemoglobin and outcomes in renal insufficiency (CHOIR) [114]

Non-dialysis patients with CKD were included and the effect of raising Hb concentration with epoetin alpha to a target Hb value of 13.5 g/dL or 11.3 g/dL was compared. The primary end point was the time of death, MI, hospitalization for congestive heart failure (excluding renal replacement therapy), or stroke.

An increased risk of the primary end point, for the high-Hb group, as compared with the low-Hb group was found. Death and hospitalization for congestive heart failure accounted for 74.8% of the events. An increased rate of thrombotic events was also reported in the group of high-Hb. Patients in the high-Hb group had a higher (but not significant) rate of both progression to renal replacement therapy and hospitalization for renal replacement therapy. They did not find any apparent additional benefit in quality of life. In conclusion, they recommended the use of a target Hb level of 11.0 to 12.0 g/dL rather than a level of 11.0 to 13.0 g/dL, because of the increased risk, increased costs, and no quality-of-life benefit.

5.1.4. Trial to reduce cardiovascular events with aranesp therapy (TREAT) [115]

In this trial patients with type 2 diabetes mellitus, CKD and anemia were enrolled. Patients were randomized to receive darbepoetin-alfa (in order to achieve a target Hb of 13.0 g/dL) or placebo (in this group were prescribed blinded "rescue" darbepoetin for Hb level < 9.0 g/dL). The primary end point was time to death or hospitalization for myocardial ischemia. A significantly higher rate of strokes in patients treated with darbepoetin was observed. A higher rate of both thromboembolism and cancer-related deaths among patients with a history of cancer in the treatment group was also reported in the treatment group.

Higher targets of Hb levels imply the use of higher ESA doses. Therefore, the increased risk for adverse CV outcomes could also result from the higher ESAs doses and not only from the normalization of Hb [116]. In this sense, a trial has been designed to identify the potential benefits and harms of different fixed doses of ESA. The Clinical Evaluation of the DOSe of Erythropoietins (C.E. DOSE) trial [117] enrolled HD patients that were randomized 1:1 to 4000 IU/week *versus* 18000 IU/week of i.v. epoietin alfa or beta, or of any other ESA in equivalent doses. The primary outcome was death, non fatal stroke, non fatal MI and hospitalization for CV causes.

Several potential mechanisms for harm with higher Hb targets have been proposed and revised by Fishbane et al. [118]. The hypothesis is that increased viscosity and hemoconcentration, the increased BP, the toxic effect of iron and unphysiological doses of ESAs contribute to ESAs toxicity. The rise in Ht results in a higher viscosity and, consequently, higher risk of thomboembolism. It also favors platelet activation by increasing the interaction between the endothelial cells and platelets in blood vessels. Hemoconcentration is a phenomenon observed in these patients after a dialysis session that results from the removal of large amounts of fluids.

5.2. Safety advisories

Considering the results of these studies, in 2007 the FDA launched a safety advisory, recommending that patients do not exceed the Hb level of 12g/dL [119]. At the same time, the NKF KDOQI made an update on its guidelines, recommending that the selected Hb target should generally be in the range of 11.0 to 12.0 g/dL, but should not be greater than 13.0 g/dL [120].

In 2010, the European Best Practice Guidelines Work Group published the recommendation that "Hb values of 11-12 g/dL should be generally sought in the CKD population without intentionally exceeding 13 g/dL" [121]. In 2011, the FDA introduced warnings in the ESA label

giving the recommendations "for more conservative dosing of Erythropoiesis-Stimulating Agents (ESAs) in patients with chronic kidney disease (CKD) to improve the safe use of these drugs" [122].

5.3. Hemoglobin variability

In conjugation with the optimal Hb target and ESA dose, there is a study of Hb variability (Hb-var). It was noted that during the treatment of HD patients with ESAs the level of Hb have a great fluctuation, that is, the Hb levels tends to rise or fall in a cyclic pattern, that is different for each patient [123]. However, the impact of this Hb-var is not still elucidated. Some studies show that there is an association between Hb-var and increase of death [11, 64, 65], especially if this variability is greater than 1g/dL [11]. The main factor for this variability is ESA dose; however, other factors have been pointed, as i.v. iron and other biological factors (inflammation and nutritional status) [123].

Hb-var represents an important physiological stress, as the ESA treatment involves short, intermittent burst of plasma EPO availability that do not coincide, either temporally or in magnitude with its physiological action. Under physiological conditions EPO levels are maintained in a narrow range, through several mechanisms, in order to support a constant oxygen supply to the organs. The impact of Hb-var on the organism is not fully understood, but the myocardium may be one of the most affected organs, as it has to compensate with an increased output and cardiomyocytes proliferation during the periods of reduced oxygen availability, that occur when Hb reaches lower levels, before the new ESA administration. This might result in deregulation of cardiac growth signal, leading to left ventricular dilation and hypertrophy [11, 123]. The autonomic nervous system can also suffer from this Hb-var; actually, autonomic dysfunction occurs in other pathological conditions, where Hb-var also occurs, like sickle cell anemia [11]. Fishane et al. also [123] found that better responders to ESA tend to have a higher degree of Hb-var.

6. Conclusion

Despite all the technologic advances in HD procedure and medical support, the morbidity and mortality in CKD patients remains high, particularly in hyporesponsiveness patients to ESAs therapy. The clinical trials showed that a higher Hb target is associated with increased risk of cardiovascular complications and death; however, the impact of higher ESAs doses to achieve higher Hb targets remains unclear. Some evidence points that the pleiotropic effects of ESAs can contribute to the ESAs toxicity observed with higher doses. Meanwhile, the recommendations to target Hb to a range of 11 – 12 g/dL, without exceeding the 13g/dL, with the lower doses of ESAs to accomplish this goal, can reduce the risks associated with higher Hb target and higher ESAs doses in CKD patients. More studies are needed on this field to evaluate the impact of the linkage anemia/high sustained ESAs therapeutic doses in CKD that might explain the high mortality in hyporesponsiveness patients. To accomplish these goals blood, cellular and tissue studies are need that cannot be performed in humans; therefore, the use of appro-

priate animal models could be useful to understand whether the association of moderate anemia and high sustained therapeutic doses of ESAs in non-responders is beneficial or an increasing risk; to clarify the underlying mechanisms and, eventually, to propose new therapeutic strategies to reduce mortality in HD patients.

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Appendix II

Iron therapy in chronic kidney disease: Recent changes, benefits and risks

Sandra Ribeiro, Luís Belo, Flávio Reis and Alice Santos-Silva

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REVIEW

Iron therapy in chronic kidney disease: Recent changes, benefits and risks

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ABSTRACT

Anemia is a common complication in patients with chronic kidney disease (CKD), mainly due to inadequate renal production of erythropoietin. In hemodialysis (HD) patients this condition may be aggravated by iron deficiency (absolute or functional). The correction of this anemia is usually achieved by treatment with erythropoiesis stimulating agents (ESAs) and iron (oral or intravenous). Studies questioning the safety of ESAs (especially at higher doses) changed the pattern of anemia treatment in CKD patients. According to the new guidelines, when transferrin saturation is lower than 30% and ferritin lower than 500 ng/mL, a trial with iron should be started, to avoid therapy with ESAs or at least to reduce the doses needed to treat the anemia. Recent reports showed increasing ferritin levels, towards values above 800 ng/mL, in CKD patients treated according to the guidelines. In this review we focus on the risks of the increased iron use to treat CKD anemia, namely, iron overload and toxicity, increased risk of infections, as well as mortality.

1. Iron disturbances in hemodialysis patients

Anemia is a common complication in patients with chronic kidney disease (CKD). The number of CKD patients with anemia increases with the progression of renal dysfunction [1]. The main cause of anemia is the inadequate production of erythropoietin (EPO), a glycoprotein mainly produced by the kidney, responsible for the growth, differentiation and reduction of apoptosis of erythroid cells in the bone marrow [2]. Iron deficiency is another common cause of anemia in these patients [3]. The high frequency of blood analysis, the surgical procedures for vascular access and the blood loss into the hemodialyser and tubes during the dialysis procedure contribute to reduce iron stores. A CKD patient under hemodialysis (HD) therapy loses about 1–2 g of iron per year through these mechanisms. Inflammation is another hallmark of CKD that may lead to a "functional" iron deficient anemia, as we and others previously reported [4–10]. Actually, inflammation has been proposed to play an important role in disturbances in iron metabolism. It is known, as we found, that CKD patients under HD present high serum levels of hepcidin [6,11], which has been described as a major regulator of body iron homeostasis. Hepcidin, synthesized by the liver, acts by inhibiting cellular iron efflux from hepatocytes, enterocytes and iron recycling macrophages, through binding to ferroportin, inducing its degradation [12]. Hepcidin synthesis is regulated by several proteins (Fig. 1), namely, hemochromatosis protein (Hfe), transferrin receptor 1 (TfR1), TfR2, hemojuvelin (HJV), bone morphogenic protein (BMP6), matriptase-2 and transferrin [13–15]. In the liver, diferric transferrin competes with TfR1 for binding to Hfe; in conditions associated with increased iron, more Hfe will be available to bind to TfR2; this complex, TfR2-Hfe, promotes HJV binding to BMP6, triggering hepcidin synthesis. Moreover, hypoxia and EPO, as well as twisted gastrulation protein 1 (TWSG1), growth differentiation factor 15 (GDF15) and, more recently, erythroferrone, all produced by erythroblasts, are also important modulators of hepcidin synthesis, inducing a downregulation of hepcidin synthesis [16,17]. Thus, HD patients under recombinant human EPO (rHuEPO) therapy might develop an iron deficient anemia due to several factors, as blood loss and inflammation.

2. Treatment of anemia

The first treatment used to correct anemia in CKD patients was blood transfusion. However, the risk of transfusion reactions (immunological sensitization), transmission of infectious agents and iron overload triggered the search for a better treatment of anemia [18].

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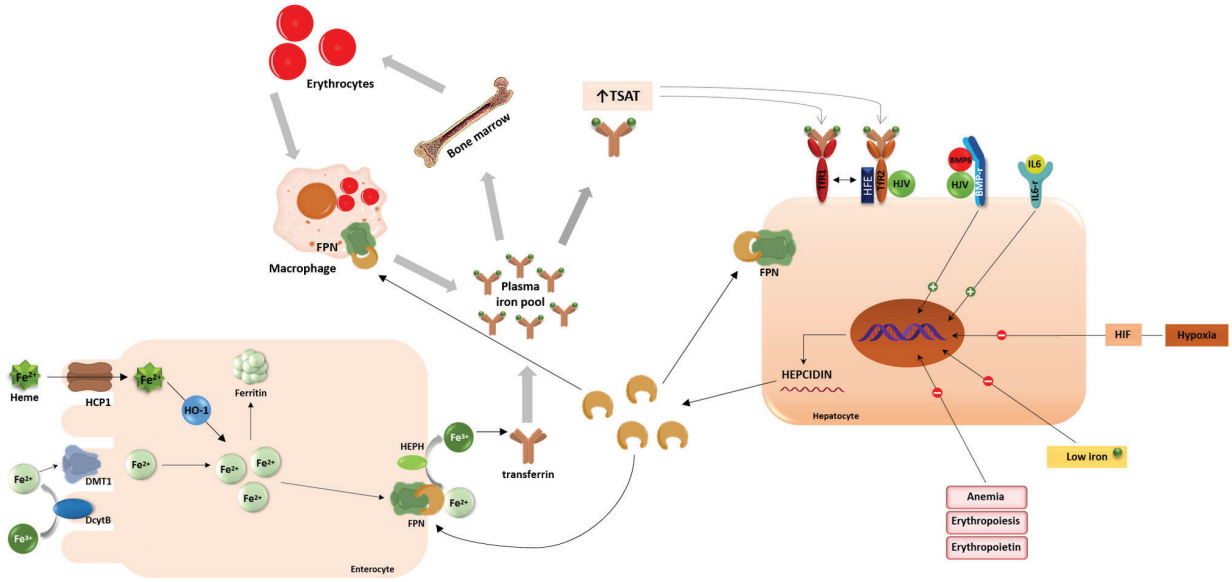

Fig. 1. Iron absorption and regulation of iron metabolism. Diet iron is present as either heme iron (Fe^{2+}) or nonheme iron (Fe^{3+}). Nonheme iron (Fe^{3+}) must first be reduced to Fe^{2+}, by duodenal cytochrome B (DcytB), before it can be transported by the divalent metal iron transporter 1 (DMT1). Once inside the enterocyte, the newly absorbed iron enters the intracellular iron pool. If the iron is not required by the body, it is loaded onto the iron storage protein ferritin. Iron required by the body is transferred across the basolateral membrane by ferroportin (FPN). The export of iron also requires the ferroxidase hephaestin (HEPH). Iron is transported by transferrin (Tf) to the local where it is needed, as in the bone marrow to erythropoiesis. The senescent erythrocytes are phagocyted by macrophages recycling iron. Hepcidin the main regulator of iron metabolism (by blocking FPN action) is synthesized by the liver and regulated by several stimulator (+) and inhibitor (−) factors, as transferrin saturation (TSAT), inflammation, anemia, erythropoiesis and hypoxia. BMP6 — Bone morphogenetic protein 6; BMP-r — BMP receptor; HFE — hemochromatosis protein; HIF — hypoxia inducible factor; HJV — hemojuvelin; IL6 — Interleukin 6; IL6-r — IL6 receptor; TFR — Transferrin receptor.

The introduction of rHuEPO in the late 80s revolutionized the treatment of anemia of CKD, particularly of end stage renal disease patients. Since then, several erythropoiesis stimulating agents (ESAs) have been approved to treat the anemia of CKD patients, differing in their half-life (Table 1), which determines the frequency and route of their administration. They were, therefore, divided into "short-acting" and "long-acting" ESAs. Epoetin beta and alpha are "short-acting" ESAs, with identical amino acid and carbohydrate composition to EPO, that are given 3 times per week. Recently, biosimilar epoetins have been introduced in the market (epoetin zeta and theta), presenting therapeutic profiles similar to those of epoetin beta and alpha [19].

The need to improve formulations, in order to reduce the frequency of administration, led to the development of "long-acting" ESAs. Darbopoetin alpha presents two additional N-linked carbohydrate chains compared to EPO, which increase its half-life and, therefore, allow its administration only once per week. In 2007, methoxy polyethylene glycolepoetin beta, a continuous EPO receptor activator (CERA) was introduced, presenting a half-life of about 130 h, which reduced its administration for once per month [20]. Peginesatide, with a longer half-life, is a pegylated homodimeric peptide with no sequence homology to EPO [21]; it was the first synthetic peptide approved for the treatment of CKD anemia, in 2012; however, due to serious hypersensitivity associated reactions, the product was removed from the market in 2013.

The introduction of ESAs reduced the need for transfusions and improved the quality of life in CKD patients, as the treatment reduced the symptoms and the comorbidities associated with anemia [22] and slowed the progression of renal disease, increasing the time before replacement therapy [23]. Although the majority of the patients respond to ESA therapy, about 10% of CKD patients are hypo-responsive [24]. To overcome this reduced response, often referred as resistance, higher doses of ESAs are needed to achieve the target hemoglobin (Hb) levels [4–6].

One of the causes for hypo-responsiveness to ESAs is iron deficiency (Fig. 2), either absolute or functional [25]. Serum iron, transferrin and ferritin concentrations, as well as transferrin saturation (TSAT), are usually used to assess iron status. Plasma/serum ferritin concentration is the only non-invasive evaluation to assess iron stores. A ferritin value lower than 30 ng/mL in men or lower than 15 ng/mL in women are consistent with absolute iron deficiency [26]; a serum ferritin concentration higher than 300 ng/mL, along with anemia, indicates a functional iron deficiency, as it usually occurs in CKD patients [6].

Serum iron and TSAT are useful to measure circulating iron, available for delivery to bone marrow erythroid cells, for Hb synthesis. In the case of anemia, it is important to distinguish between absolute iron deficiency (low levels of iron, ferritin and TSAT), which usually is responsive to iron therapy, from a functional iron deficiency (high levels of ferritin and low iron and low/normal levels of TSAT) that results from an inflammatory state. Indeed, the inflammatory condition triggers the production of hepcidin, which inhibits iron absorption and mobilization, leading to the development/worsening of anemia in CKD patients [27].

Nowadays, the gold standard treatment for anemia in CKD patients is the administration of ESAs, associated with iron supplementation. When ESA therapy was introduced, the patients were treated to normalize hematocrit (Ht) and Hb levels. However, the first clinical trial evaluating the outcomes of the patients, according to their Ht, showed that values higher than 42% were associated with increased number of deaths [28]. The publication of other three studies [29–31] reporting similar conclusions raised concerns about the risks associated with normalization of Ht, and about the risks associated with the higher ESA doses needed to achieve those Ht values. Considering the results of these studies, the Food and Drug Administration (FDA) launched in 2007 some safety advisories, recommending that patients should not exceed the Hb level of 12 g/dL [32]. Later, in 2011, the use of more

Table 1
Erythropoiesis-stimulating agents approved for CKD anemia treatment.

Short-acting	Long-acting
Epoetin beta	Darbopoetin alpha
Epoetin alpha	Methoxy polyethylene glycolepoetin beta
Epoetin zeta	
Epoetin theta	

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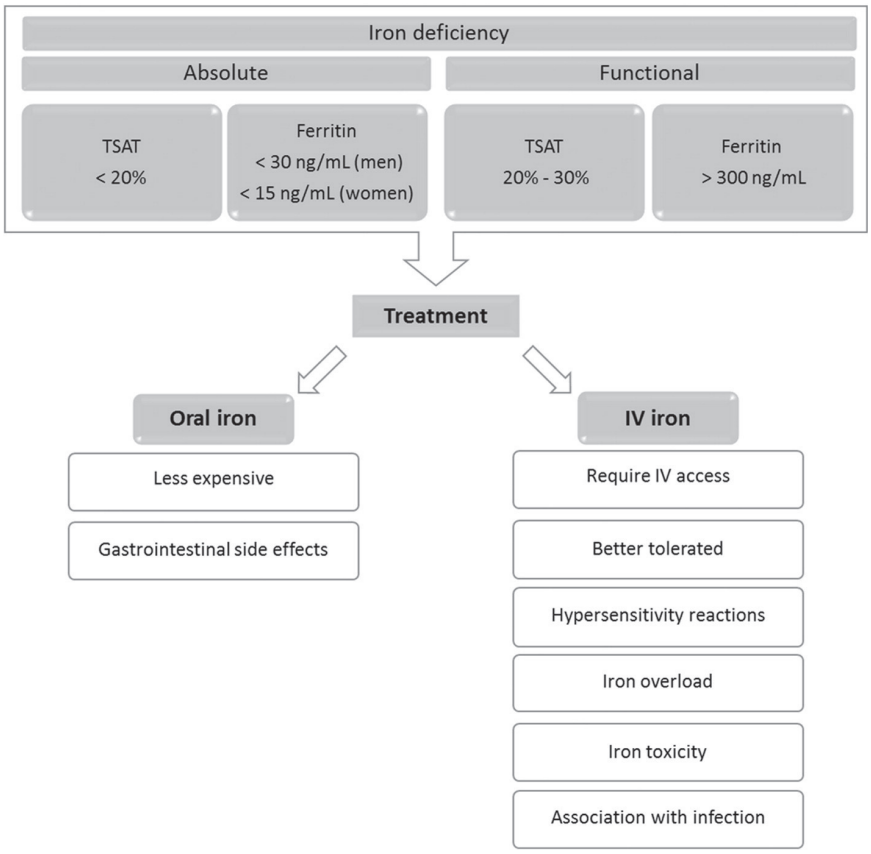

Fig. 2. Iron deficiency and treatment. Iron deficiency can be absolute or functional according to transferrin saturation (TSAT) and ferritin values. Oral or intravenous (IV) iron can be used to correct iron deficiency depending on patient characteristics.

conservative ESAs doses was recommended [33]. In accordance, the Kidney Disease Improving Global Outcomes (KDIGO) and the European Best Practice Guidelines (EBPG) Work Group made also an update to their guidelines, recommending that Hb levels should be 11–12 g/dL, without intentionally exceeding 13 g/dL and that a safer use of ESAs should be adopted [24,34]. Moreover, the "Clinical Practice Guideline for Anemia in Chronic Kidney Disease" [24] from KDIGO also stated that CKD patients with anemia should first start iron therapy rather than ESAs. They suggested that CKD patients should start iron supplementation when TSAT is lower than 30% and ferritin levels lower than 500 ng/mL; however, the upper safe serum ferritin level was not defined.

The use of iron is now a common practice in the treatment of anemia in CKD patients, in spite of the concerns about the increase in ferritin levels and tissue iron overload, due to the use of high/continuous iron supplementation.

3. Oral and intravenous iron

Supplementation of HD patients with iron is needed to maintain iron available for an adequate erythropoiesis. The decision for oral or intravenous (IV) iron treatment should balance the benefits and risks for the patient (Fig. 2).

Table 2
Iron supplements approved for CKD anemia treatment.

Oral	Intravenous
Ferrous sulfate	Iron sucrose
Ferrous gluconate	Ferumoxytol
Ferrous fumarate	Ferric carboxymaltose
	Iron dextran

Oral iron formulations (Table 2) are less expensive; however, the gastrointestinal side effects experienced by some patients (about 30%) may reduce the effect and adherence to treatment [35,36]. Some studies reported that IV iron therapy increases both Hb and ferritin, while oral iron therapy increases Hb without increasing iron stores [35,36]. Moreover, most of the clinical studies evaluating oral and IV administration reported that IV iron therapy leads to a higher increase in Hb than oral iron therapy [36,37].

IV iron therapy is better tolerated than oral iron therapy [35,36], has more adherence and efficacy, but requires the existence of an IV access. It has been associated with hypersensitivity reactions that, although very rare, can be life-threatening [38]. The major risk factors for these hypersensitivity reactions include a previous reaction to an iron infusion, a fast iron infusion rate, multiple drug allergies, severe atopy and systemic inflammatory diseases [38].

IV iron therapy is preferred for patients under HD, as they have a vascular access already available that can be used for iron infusion. In non-dialysis (ND) CKD patients, oral iron may be preferred to preserve IV access. However, there is no consensus for the use of oral rather than IV iron as first line treatment in ND-CKD patients. Several studies have been performed [35,37,39,40] to further clarify the efficacy and safety of oral versus IV iron supplementation in ND-CKD patients. Stoves et al. [39], in a follow-up study along 6 months of ND-CKD patients, found that the Hb response and ESA requirements with IV iron (iron sucrose) or with oral iron (ferrous sulfate) supplementation were similar. In accordance, Charytan et al. [35] and Agarwal et al. [41] reported that both IV and oral iron therapies in ND-CKD patients resulted in similar Hb responses, but IV iron therapy showed better results in replacing

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iron stores. Conversely, Qunibi et al. [36], Van Wyck et al. [37] and the recently FIND-CKD study [40] found that IV iron is superior to oral iron therapy, as ND-CKD patients under IV iron therapy showed higher Hb levels and iron stores, as well as a higher improvement in their quality of life.

The efficacy of oral iron therapy can be compromised in CKD, as these patients usually present a low to mild degree of inflammation, which leads to an increase in hepcidin levels [6,42]. This increase in hepcidin, by reducing gastrointestinal iron absorption and decreasing iron release from body storage sites, inhibits the use of iron for erythropoiesis and leads to an increase in ferritin (Fig. 1).

One of the concerns in IV iron therapy is that the used iron dose overcomes the binding capacity of transferrin, leading to an increase in non-transferrin bound iron (NTBI). Moreover, as transferrin levels are usually reduced in CKD patients, this effect might be enhanced [6,43]. The presence of NTBI can lead to the development of oxidative stress, increasing the pro-oxidant state of these patients. In the case of co-existence of inflammation and cardiovascular (CV) disease, the oxidative stress may be even more enhanced. It has been also suggested that NTBI might be a source for iron deposition in organs [44–46].

The optimization of iron delivery, given the complications associated with IV iron therapy and the reduced oral iron adherence and efficacy, is an actual challenge to search and develop better iron formulations (Table 2). Recently, a treatment with oral liposomal iron showed to be a safe and efficacious alternative to IV iron in ND-CKD patients [47]. Iron-carbohydrate complexes (IV formulations), presenting lower hypersensitivity reactions than iron-dextran complexes and allowing the gradual release of iron (instead of a cumulative iron dose), have been developed and approved [48].

3.1. Iron overload

Iron has essential roles in several physiological processes, including the synthesis of Hb and other hemeproteins. The body iron stores are tightly controlled, as there is no physiological mechanism of iron excretion. Iron can be stored in the body, as ferritin and hemosiderin. These forms are found in the macrophages of the reticuloendothelial system (liver, spleen and bone marrow) and in the parenchymal liver cells. Serum ferritin, TSAT and reticulocyte count are used to evaluate iron stores and bone marrow iron availability. Ferritin levels can be increased in the case of inflammation and do not seem to be correlated with hepatic iron overload [49]. The first test used to evaluate iron availability to the bone marrow was performed by biopsy; as it is an invasive test, associated with some risks, it is used only to study specific iron disorders. The non-invasive techniques, as magnetic resonance imaging and magnetic susceptometry, are new tools to evaluate iron overload in CKD patients. However, these techniques do not distinguish between non-bound iron and iron stored in the macrophages of the reticuloendothelial system. Nowadays, the challenge is to find new markers and better techniques to predict iron overload [50].

The Dialysis Outcomes and Practice Patterns Study (DOPPS) [51] showed that the number of HD patients with IV iron supplementation increased in most countries; however, and even more important, the doses prescribed to these patients also increased in the past 10–15 years. Overall, the mean serum ferritin has increased over time, and the proportion of patients with serum ferritin values ≥800 ng/mL has also increased [51].

The recent KDIGO Controversies Conference on Iron Management in CKD (San Francisco, 2014) recognized the entity of iron overload in HD patients. Actually, there are several studies reporting tissue iron accumulation in HD patients treated with ESAs and IV iron (Table 3) [49,52–54]. Canavese et al. [52] found that 70% of HD patients presented mild-to-severe hepatic iron overload, although the same percentage of patients presented serum ferritin values below 500 ng/mL. Ferrari et al. [49] reported that 60% of HD patients presented liver iron concentration >60 μmol/g (above the normal upper limit of 30 μmol/g) and 13% had liver iron concentrations >130 μmol/g, which is usually found in patients with hemochromatosis; they also found that iron liver accumulation was correlated with cumulative iron dose, but no correlation was observed with ferritin or TSAT. Recently, two other studies found similar results [53,54]. Ghoti et al. [53] reported that 90% of HD patients presented mild to severe hepatic iron deposition and also iron deposition in the spleen. In the other study, 84% of the HD patients had hepatic iron overload, and in 30% of them iron overload was severe; moreover, iron liver content correlated with infused iron [54].

In spite of the widespread use of IV iron supplementation in HD patients, the safest dosing strategy is still poorly clarified, as well as its relation with serum ferritin levels, iron overload and mortality risk. Actually, only few studies addressed these questions, and presented controversial results (Table 3). Feldman et al. [55] found that HD patients receiving more than 1000 mg of IV iron in a period of 6 months presented a higher risk of death and hospitalization, compared to patients with no iron supplementation. In a follow-up study of 2 years, the same group evaluated the effect of each 6 months of iron exposure and did not find any association between the iron administrated and mortality, but found an association between iron dose and mortality at 12 to 18 months of treatment, for iron doses higher than 1800 mg [56]. In the first study of this group [55], only 17% of the HD patients received iron dosing above 1000 mg, whereas in the second study [56] this number rose to 48%. These authors also found an association between mortality and ferritin levels >800 ng/mL in the 6 months prior to death, but this finding could result from confounding factors, such as inflammation and malnutrition [56].

Another observational study evaluating HD patients for a period of 2 years showed that serum ferritin levels in the range of 200–1200 ng/mL, TSAT between 30 and 50% and IV iron dose <400 mg by month were associated with improved survival, suggesting that the association of high ferritin levels and mortality could also result from confounding factors [57]. A recent study [58] evaluated the effect of a bolus of IV iron (consecutive doses ≥100 mg exceeding 600 mg during one month) versus maintenance of IV iron therapy (all other iron doses during the month), and also the effect of high (>200 mg over 1 month) versus low dose of IV iron (≤200 mg over 1 month); after 1 month of iron exposure, the patients were studied for a follow-up period of 3 months; no significant associations of bolus versus maintenance dose or of high versus low dose IV iron with increased short-term CV morbidity and mortality were found.

Zitt et al. [59] found that increasing ferritin concentrations (>800 ng/mL) in patients with normal C-reactive protein (CRP) concentrations were associated with decreasing mortality, whereas in patients with high CRP (>0.5 mg/dL) values the increasing ferritin concentration was linked with increased mortality, suggesting that serum ferritin levels above 800 ng/mL are associated with increased mortality in the case of concomitant inflammation. A 2-year follow-up study, by our group [6], found that HD patients presented several changes in iron metabolism (increased TSAT, ferritin and hepcidin and a decrease in iron and transferrin) and inflammatory markers (increased interleukin [IL]-6 and CRP). Patients who died during this follow-up period showed significantly lower values for transferrin and TSAT and increased levels of IL-6 and CRP. In the adjusted survival regression model, CRP was found to be a significant predictor of mortality.

The recently published observational DOPPS study [60] showed that HD patients receiving, along 4 months, 300 mg/month or even a higher dose of IV iron supplementation, compared with patients supplemented with 100–199 mg/month, presented increased hospitalization risk, all-cause mortality and CV mortality, regardless of serum ferritin or CRP concentrations.

Considering the controversial data in literature, there is a clear need to develop clinical trials with longer follow-up periods of HD patients to evaluate the effect of long-term cumulative IV iron doses and the impact of serum ferritin levels on all-cause and CV mortality. In accordance, a clinical trial, "The Proactive IV irOn Therapy for HaemodiALysis patients (PIVOTAL)", has recently started, and the aim is to compare the effect of

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Table 3
Studies evaluating iron overload and mortality risk associated with intravenous iron therapy in dialysis patients.

	Year	Patients	Iron dose/follow-up time	Outcome	Ref.
Iron overload	2012	HD patients (n = 119)	100 mg of iron sucrose (2–3×/w — induction phase; 1×/w every 4 w — maintenance phase) 60 mo follow-up	• 84% of patients with hepatic iron deposition, 30% of them with severe iron overload • Iron liver content correlates with infused iron	[54]
	2012	HD patients (n = 21)	100 mg (ferric saccharate) 1–3×/w 12 mo follow-up	• 90% of patients with mild-to-severe hepatic iron deposition • 95% of patients with splenic iron deposition • 14% patients with pancreatic iron deposition	[53]
	2011	CKD patients (n = 25)	50 to 200 mg/mo for 12 mo	• Liver iron concentration: 60% of patients >60 μmol/g; 13% of patients >130 μmol/g (reference value 30 μmol/g)	[49]
	2004	HD patients (n = 40)	31.25 mg ferric gluconate complex 10 patients: maintenance iron at least 6 mo 30 patients: without iron therapy at least 2 mo (after ferritin >500 μg/L)	• 70% of patients with mild-to-severe hepatic iron overload • 70% of patients with ferritin <500 ng/mL	[52]
Mortality	2015	HD patients (n = 32,435)	4 mo-follow up of IV iron dose: <300 mg/mo versus ≥ 300 mg/mo	• ↑ mortality among patients with higher doses of IV iron (≥300 mg/mo over 4 mo), regardless serum ferritin or CRP values	[60]
	2014	Dialysis patients (n = 235)	7.6 year-follow up with continuous maintenance iron therapy once/w in varying doses: 12.5 mg (minimum dose) to 62.5 mg (maximum dose)	• ↑ mortality with ferritin levels >800 ng/mL in the case of concomitant inflammation (CRP > 0.5 mg/dL)	[59]
	2013	HD patients (n = 117,050)	3 mo-follow up after 1 mo exposure to: high dose (>200 mg) versus low dose (1–200 mg) bolus (consecutive doses ≥100 mg exceeding 600 mg during one month) versus maintenance dose	• No significant associations of bolus versus maintenance dose or high dose versus low dose IV iron with increased short-term cardiovascular morbidity and mortality	[58]
	2005	HD patients (n = 58,058)	Iron gluconate effect in a 2 year-follow up, with different iron dose categories: 0 mg/mo; 1 to 199.9 mg/mo; 200 to 399.9 mg/mo; >400 mg/mo	• Ferritin 200–1200 ng/mL, TSAT 30–50% and IV iron dose <400 mg/mo associated with improved survival	[57]
	2004	HD patients (n = 27 280)	Effect of iron administration in a 2 year follow-up, with iron doses categories at each 6 mo: >0 to 700 mg; >700 to 100 mg; >1000 to 1800 mg; >1800 mg	• No association between iron administrated and mortality • Association between iron dose and mortality at 12 to 18 mo of treatment, for doses >1800 mg • Association between mortality and ferritin >800 ng/mL in the 6 mo prior to death	[56]
	2002	HD patients (n = 10 169)	Number of 100-mg vials of iron during 6 mo	• ↑ risk of death and hospitalization with IV iron >1000 mg	[55]

CRP — C reactive protein; HD — hemodialysis; IV — intravenous; mo — month; w — week.

IV iron high-dose versus low-dose regimen on all-cause mortality, and to evaluate the incidence of non-fatal CV endpoints, in HD patients along 2–3 year follow-up [61]. Another aim of this study is to compare the effect of the two regimens on ESA dose requirements, red blood cell transfusions, complications of HD treatment, and quality of life of the patients.

3.2. Iron toxicity

Data in literature strongly suggest the risk of tissue iron overload in HD patients, although the underlying pathophysiological mechanisms are less clear, especially in the case of IV administration of iron. The infusion of iron may overwhelm the capacity of the iron binding proteins, allowing iron to become free in circulation and/or to increase iron stores. It is known that free iron can react with hydrogen peroxide leading to the production of hydroxyl radicals that are able to trigger oxidative modifications in lipids, proteins and DNA. Indeed, the literature supports that after IV iron injection in HD patients a transient increase in oxidative stress occurs, as shown by the increase in plasma lipid peroxidation [62] and oxidative modification of proteins [63]. Actually, different markers of oxidative stress are significantly increased in CKD patients [64,65] and are involved in the progression of renal disease [66].

Several diseases have been associated with oxidative stress, such as the CV diseases [67,68], which are the major cause of death in HD patients. It has been hypothesized that the oxidative stress induced by IV iron infusion could favor atherosclerosis and endothelial cell damage. Reis et al. [69] found that in dialysis patients the carotid intima media thickness correlated with serum ferritin and with the IV iron dose, corroborating the previous hypothesis. Studies in rabbit models found that the more extensive atherosclerotic lesions contained a higher concentration of iron and that the formation of these lesions may be accelerated by free radical production, caused by increased iron levels [70,71]. Kuo et al. [72] reported that CKD patients who had received IV iron sucrose preparation presented increased superoxide production by circulating mononuclear cells and increased expression of soluble adhesion molecules and of mononuclear-endothelial adhesion molecules, when compared with untreated CKD patients or healthy subjects. In the same study, the authors corroborated their results by showing that mice with uninephrectomy treated with IV iron presented increased tissue superoxide production, increased expression of tissue cell adhesion molecules, endothelial adhesiveness and exacerbated atherosclerosis in the aorta. These effects seem to be dependent on IV iron formulation used, as reported by Toblli et al. [73].

NTBI might be important for extrahepatic iron deposition and toxicity, namely, in the kidney. Progressive tubulointerstitial damage and renal fibrosis are common pathways in the development of CKD and iron deposition could favor these lesions. Indeed, iron accumulation is observed in the proximal tubule in human CKD [74], as well as in rats with nephropathy [75–78], and seems to be associated with the progression of CKD. In a study by our group, using a rat model of nephrectomy, we found iron deposition in tubules, along with extensive tubulointerstitial lesions [78]. Recently, in a similar model, Naito et al. [76] reported that the animals with renal failure, treated with oral iron

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chelators, showed a reduction in interstitial fibrosis, suggesting that renal iron accumulation is associated with renal interstitial fibrosis, promoting the progress of renal disease.

3.3. Association of iron therapy with infection

In vitro studies show a relationship between the availability of iron and bacterial virulence, as iron is important for bacteria multiplication in the host. Therefore, clinical conditions associated with iron excess in the host may increase the risk for infection [79]. Clinical studies reported different results about the linkage of IV iron therapy with infection. One study observed a significantly higher rate of bacterial infection with higher IV iron saccharate dose, but not with higher frequency of dosing administration [80]. A one year follow-up study of HD patients examined the relationship between iron stores, IV iron dosing and bacteremic risk, and found that patients with higher iron stores had a significantly higher risk of bacteremia; however, they did not find an IV iron dose–response relationship [81]. Brewster et al. [82] reported that IV iron did not significantly increase the rate of microorganism growth within catheters or the development of blood infections with iron supplementation. After the recent changes in the pattern of IV iron treatment [24], Bansal et al. [83] in a 2 year follow-up study of HD patients treated with IV iron found a significant increase in TSAT and in serum ferritin; however, it was not associated with an increase in the incidence of infectious complications. In a retrospective cohort study of HD patients, those receiving bolus versus maintenance iron therapy were at increased risk of infection-related hospitalization [84]. Recently, Kuragano et al. [85] found that patients with a higher ferritin level had a higher risk of infectious disease than those with lower ferritin level. They also found that the risk of infection and hospitalization were significantly higher among patients who were treated with high weekly doses of IV iron, compared with no iron. An observational study that included 14,078 patients reported the possibility of infection-related mortality with higher iron doses [86].

The type of IV iron formulation might have also an impact in the rate of infection, as suggested by Sirken et al. [87], that found a higher bacteremia rate with iron sucrose than with ferric gluconate; however, a relationship between IV iron dose and bacteremia was not supported. Randomized clinical trials are needed to assess the effect of cumulative IV iron doses and the risk of infection-related mortality.

4. Conclusions

The new protocol for anemia management, in accordance with the recent published guidelines "Clinical Practice Guideline for Anemia in Chronic Kidney Disease" from KDIGO [24], contributed to an increase in the frequency and in the dose of iron used to treat anemia of CKD patients. Higher values of TSAT and ferritin have been found in dialysis patients, raising concerns about iron overload. Data reported in literature alerts to the safety of cumulative higher iron doses in dialysis patients, as they seem to be associated with increased risk of iron overload, toxicity and infection, which can lead to an increased risk of mortality in these patients. Actually, there is a need to clarify the effect of the actual iron dosing in CKD patients and its effect on mortality.

Practice points

- Iron deficiency (absolute or functional) is a common cause of anemia in CKD patients.
- The number of HD patients with IV iron supplementation and the prescribed doses increased in the past years.
- The prevalence of HD patients with ferritin levels above 800 ng/mL has increased.
- Iron overload in HD patients has been reported in several studies; however, the relationship between iron overload and mortality risk is not clear.
- Serum ferritin is not always a predictor of iron overload, due to confounding factors, as inflammation and malnutrition.
- Non-invasive techniques, as magnetic resonance imaging and magnetic susceptometry, are new tools to evaluate iron overload in CKD patients.

Research agenda

- Clinical trials to evaluate the association between high iron doses, iron overload and mortality risk in HD patients.
- Basic and clinical research is still needed to find better markers and assays to evaluate iron overload.

Conflict of interest statement

The authors report no conflicts of interest.

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